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TITLE: EXTERNAL FACTORS, PRODUCED BY GROWING NERVES, TRIGGER A
REGENERATIVE RESPONSE IN A NONREGENERATIVE CENTRAL
NERVOUS SYSTEM: PURIFICATION AND MODE OF ACTION

PRINCIPAL INVESTIGATOR: Michal Schwartz, Ph.D.

CONTRACTING ORGANIZATION: Weizmann Institute of Science
76100 Rehovot
Israel

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TABLE OF CONTENTS

	Page
Report Documentation Page (DD Form 1473)	1
Foreword	2
Abbreviations	2
1. Statement of the problem	3
1.1. The general problem and the significance of this research	3
1.2. The basic research problem	3
2. Background	4
2.1. Environmental elements and regeneration	4
2.2. Diffusible substances and nerve regeneration	6
2.3. Extracellular matrix components and nerve regeneration	7
2.4. A regeneration-like response in a nonregenerative system can be triggered by diffusible substances originating from growing nerves	8
2.4.1. Growth in GATFs-treated injured nerves	9
2.4.2. GATFs include factors which activate glial cells	9
2.4.3. GATFs include factors which inhibit maturation of oligodendrocytes	11
3. Rationale for the approach taken in this work	11
4. Techniques	14
4.1. Surgical procedures	14
4.1.1. Carps	14
4.1.2. Rabbits	14
4.2. Preparation of conditioned media	15
4.3. Application of horseradish peroxidase	15
4.4. Immunocytochemical analysis for laminin immunoreactive sites of nerve sections	16
4.5. <u>In vitro</u> approach for laminin production	16
4.5.1. Treatment of C-6 cells	16
4.5.2. ELISA screening methods for surface antigens	17
4.6. Two-dimensional gel electrophoresis	17
4.7. Immunoblot	18
4.8. Solid phase radioimmunoassay	18
4.9. Immunocytochemical localization at the electron microscope level	18
4.9.1. Fixation	18
4.9.2. Electron microscopy	19
4.10. Cultures of primary brain oligodendrocytes	20
4.11. Assessment of heparin binding activity of apolipoproteins	20
4.11.1. Determination of affinity to protein	20
4.11.2. Solid phase radioassay	21
4.11.3. Reversibility of [³ H]heparin binding to apolipoprotein-A-I	21

	Page
5. Results	22
5.1. Purification of apolipoprotein-A-I from regenerating fish optic nerve and its characterization	22
5.1.1. Isolation of apo-A-I from CM of regenerating fish optic nerves	23
5.1.2. Analysis of heparin binding capacity of apo-A-I	24
5.2. GATFs include factor(s) which block differentiation of oligodendrocytes from their progenitors	27
5.3. Qualitative and quantitative analyses of the growth induced into the injured adult rabbit optic nerves	32
5.3.1. Normal rabbit optic nerve	32
5.3.2. Transected rabbit optic nerve (operated control)	33
5.3.3. Transected rabbit optic nerves subjected to combined treatment	34
5.3.4. Origin of the unmyelinated and thinly myelinated axons	37
5.3.5. Quantitative analysis of axons in nerves at 2 to 8 weeks postoperatively in treated and operated control nerves	38
6. Conclusions	39
References	41

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LIST OF TABLES

	Page
Table I: NH ₂ -terminal amino acid sequence	48
Table II: Yield of apo-A-I isolated from media conditioned by regenerating fish optic nerves	49
Table III: Comparison of periodically of myelin lamellae in axons associated with CNS glial cells and axons associated with Schwann cells	50
Table IV: Comparison of number of axons in treated nerves 8 weeks postoperatively, at two levels distal to the site of injury	51
Table V: Comparison of myelin index in unoperated control nerves and in treated nerves, 8 weeks postoperatively	52
Table VI: Comparison of diameters of myelinated axons in unoperated controls and treated nerves, 8 weeks postoperatively	53
Table VII: Comparison of numbers of viable axons in operated control and in treated nerves 2 mm from site of injury, at 2-8 weeks postoperatively	54

LIST OF FIGURES

	Page
Figure 1: A three-step purification of apo-A-I from fish CM	55
Figure 2: Analysis by SDS-PAGE of apo-A-I in the various steps of its purification from CM	55
Figure 3: Heparin binding of apo-A-I within media conditioned by regenerating fish optic nerves	55
Figure 4: Heparin binding of apo-E in media conditioned by regenerating sciatic nerves	56
Figure 5: Heparin binding of purified apo-A-I derived from fish plasma	56
Figure 6: Heparin binding of apo-A-I derived from human plasma	56
Figure 7: Heparin-bound [125 I]apo-A-I derived from fish CM	57
Figure 8: Binding assay of apo-A-I to [3 H]heparin	57
Figure 9: Effect of fish CM of regenerating fish optic nerves on adult O-4 positive cells in cultures of adult injured pc-3 rat optic nerves	57
Figure 10: The effect of CM of regenerating fish optic nerves on <i>in vitro</i> development of galc positive cells from 1 day postnatal rat brain	58
Figure 11: Comparison between effects of PDGF and CM of regenerating fish optic nerves on neonatal rat brain oligodendrocytes	59
Figure 12: The combined effect of CM fish optic nerves and PDGF on development of galc positive cells	59
Figure 13: Comparison between the effect of CM of regenerating and intact fish optic nerves on development of positive cells in cultures of rat brain oligodendrocytes	59
Figure 14: CM of regenerating fish optic nerves affect selectively oligodendrocytes in mixed newborn rat cultures of astrocytes and oligodendrocytes	60
Figure 15: Electron micrograph of a cross section through optic nerve of a normal adult rabbit	61

	Page
Figure 16: Electron micrographs of operated nontreated control adult rabbit optic nerve, 2 mm distal to the site of the injury	61
Figure 17: Reconstruction of an operated control adult rabbit optic nerve, 8 weeks postoperatively	62
Figure 18: A diagram of a cross section of a treated injured rabbit optic nerve, 6 weeks postoperatively	62
Figure 19: Electron micrographs showing characteristics of a treated injured adult rabbit optic nerve 2 mm distal to the site of injury, 6 weeks postoperatively	62
Figure 20: Some of the characteristics of axons in treated injured adult rabbit optic nerve	63
Figure 21: Comparison between myelinated axons associated with Schwann cells and those associated with putative glial cells	63
Figure 22: Postembedding immunocytochemistry using antibodies against GFAP	63
Figure 23: Postembedding immunocytochemistry using GFAP antibodies	64
Figure 24: Reconstruction of treated optic nerve 8 weeks postoperatively	64
Figure 25: Quantitative analysis of axons in the viable compartment of treated injured adult rabbit optic nerve, 1.5 mm distal to the injury	64
Figure 26: Analysis of axons in the compartment of viable axons at a level of 3.5 mm distal to the injury in treated injured adult rabbit optic nerve (Fig. 24, section 6)	65
Figure 27: Comparison between myelinated axons in unoperated control nerves and treated injured nerve	65
Figure 28: Distribution of diameter and myelin indices of axons in Figure 27b,c	65
Figure 29: HRP-labeled axons in treated injured and control adult rabbit optic nerves	65

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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ABBREVIATIONS

Apo-A-I	- Apolipoprotein-A-I
Apo-E	- Apolipoprotein-E
CM	- Conditioned media
CM-N	- Conditioned media of intact optic nerve
CM-R	- Conditioned media of regenerating optic nerve
CNS	- Central nervous system
FCS	- Fetal calf serum
Galc	- Galactocerebroside
GFAP	- Glial fibrillary acidic protein
NGF	- Nerve growth factor
PDGF	- Platelet-derived growth factor
PNS	- Peripheral nervous system

1. STATEMENT OF THE PROBLEM

1.1. The general problem and the significance of this research

The nerves of the mammalian central nervous system very rarely repair themselves after injuries which comprise a considerable proportion of combat casualties and are the most common cause of severe permanent posttraumatic neuromotor paralysis. These injuries, which include brain, spinal cord and major sense organ trauma, cause permanent disability because the adult mammalian central nervous system (CNS) is incapable of functional regeneration. Due to the poor regenerative capacity of mammalian CNS, an injury in the lower spinal cord often results in the permanent confinement of the casualty to a wheel chair. Laceration in the upper spinal cord leads to paralysis of the four limbs and a cut in the optic nerve leads to complete incurable blindness. Furthermore, the inability of the central nervous system to regenerate is the main reason why reparative and transplantation surgery in the CNS can not be performed.

Means which will induce central nervous system regeneration will be of immense benefit to soldiers suffering from these common combat injuries of the head, spinal cord and major sense organs.

1.2. The basic research problem

The process of axonal regeneration involves (i) establishment of a neuronal growth state and formation of new sprouts; (ii) elongation of new sprouts; and (iii) target recognition, synaptogenesis and cessation of axonal elongation. Successful regeneration probably depends on a synchronized progression of the events that are involved in these major phases. Malfunction of any of these events or lack of synchrony may hamper or inhibit the process of regeneration.

Our studies as well as studies carried out in other laboratories

provide evidence that the nature of the environment of the injured nerves has a key role in regeneration. Our studies on regeneration have, therefore been focused on identifying the environmental elements which are involved in the response to injury (cellular, soluble and extracellular nonsoluble). The information from these studies should provide ways to devise various means of intervention after CNS injury to facilitate regeneration such as: (i) supply of neurotrophic factors which may save neurons from secondary cell death or which activate neurons to grow; (ii) provide glial cells with growth factors which may modulate their response to the injury and thereby allow them to acquire growth supportive features, such as production of laminin or other substances needed for growth; (iii) identification of other growth modulating factors such as proteases inhibitors or lipid mediators; and (iv) search for a combined treatment.

2. BACKGROUND

Recent studies carried out in various laboratories including our laboratory provide evidence that the environment which surround the injured nerve has a significant influence on determining the regenerating capacity of the nerve.

The emphasis in our study of regeneration of CNS neurons is therefore focused on identifying the factors which allow and induce the neurons to regenerate and on attempting to devise ways to circumvent the impediments to central nerve regrowth.

2.1. Environmental elements and regeneration

As a result of injury, the neuron is deprived from target-derived substances and its mutual relationship with the environment is disrupted. The state of growth of the surrounding nonneuronal cells is altered.

(Nathaniel and Nathaniel, 1973; Nathaniel and Nathaniel, 1981; Nathaniel and Pease, 1963; Neumann et al., 1983a; Stevenson and Yoon, 1978). This may lead to formation of an environment hostile to regeneration due to the appearance of either a scar tissue (composed of collagen or glia) or axonal growth inhibitors (McConnell and Berry, 1982). Alternatively, the glial proliferation may lead to formation of a growth-supportive environment (Manthorpe et al., 1983; Schwartz et al., 1985; Varon et al., 1983; Williams and Varon, 1983). It appears that the formation of both the hostile and the supportive environments may occur at different time periods after injury (Neumann et al., 1983). It is the net outcome of these opposing contributions which may have an impact on the nature of the response to the injury, i.e., regeneration or degeneration.

Surgical manipulations were performed to determine whether a dense glial scar which is formed by the proliferating glial cells interferes with outgrowth of neurites in regeneration. These studies showed that such a scar does not represent a major obstacle to axonal growth (Reier, 1979; Reier et al., 1983). However, there is no conclusive information as to the contribution of scarring to the failure of regeneration in mammalian CNS (Billingsley and Mandel, 1982; Krikorian et al., 1981; Molander et al., 1982).

Nerve transplantation experiments demonstrated that the neuronal environment must be conducive for regeneration (Aguayo et al., 1978; David and Aguayo, 1981; Kao et al., 1977; Richardson et al., 1982). Injured spinal cord and brain axons regenerate readily through grafted columns of Schwann cells in peripheral nerves but seldom enter grafted CNS nerve segments (Aguayo et al., 1978; Kao et al., 1977; Richardson et al., 1982). This indicates that in contrast to glial cells, the nonneuronal cells of the peripheral nerves (e.g., the Schwann cells) or

components associated with them (e.g., diffusible or extracellular matrix) have the appropriate properties for regeneration. The contribution of these elements to regeneration is summarized below.

2.2. Diffusible substances and nerve regeneration

Axonal injury causes changes in the activity of diffusible substances originating from the nonneuronal environment and also from the target organ. Thus, for example, the growth of the proximal stump of a transected PNS nerve is facilitated by diffusible proteinaceous molecules possibly anchored in the basal lamina and released from the distal stump of the transected nerve (Longo et al., 1983). Similarly, axonal injury induces increased neurite-promoting activity in extracts of the target organ (Giulian et al., 1986; Henderson et al., 1983; Nieto-Sampedro et al., 1984; Nurcombe et al., 1984). Furthermore, extracts prepared from denervated adult skeletal muscle contain an increased amount of neurotrophic activity which promotes survival of dissociated motor neurons and outgrowth of neurites from explants of spinal cord maintained in serum-free defined media (Nurcombe et al., 1984). This injury-induced increase in activity has also been observed in the brain. For example, the activity of a diffusible substance which is collected from the site of a brain lesion and which affects the survival of chick sensory neurons in culture is increased after lesion (Nieto-Sampedro et al., 1983). Injury-induced increased activity is also manifested by the better survival of brain grafts in wound cavities several days after the injury (Nieto-Sampedro et al., 1983; Nieto-Sampedro et al., 1984).

Injury to optic nerves of fish (Rachailovich and Schwartz, 1984) and peripheral nerves of mammals (Skene and Shooter, 1983) both regenerative systems, causes changes in type and amount of diffusible substances

derived from surrounding nonneuronal cells. We hypothesized that among the various polypeptides which show variation in regeneration are components which are involved in glial modulation for regeneration. This issue is further discussed below.

2.3. Extracellular matrix components and nerve regeneration

Extracellular matrices have been shown to have numerous functions in attachment and migration of cells during development and regeneration. Thus, the ability of axons to grow through tissue in vivo during development and regeneration may be regulated by the availability of extracellular matrix components. Among the various components laminin was found to have the most significant effect. Laminin is known to be a key macromolecule in supporting growth and elongation of central and peripheral nervous system neurons both in vivo and in vitro. It has been proposed that the continuous expression of laminin by astrocytes is a prerequisite for axonal growth and regeneration in adult CNS (Liesi, 1985; Hopkins et al., 1985). Laminin appears in immature brain cells during CNS development and its presence coincides with phases of neural migration (Liesi, 1985; Liesi et al., 1984a,b). In adult brain laminin has been detected only in association with capillary walls and meningeal structures in contrast to its widespread distribution in the peripheral nervous system (McCloon, 1986; Cornbrooks et al., 1983; Bunge et al., 1982). Thus, in vivo adult astrocytes do not ordinarily produce laminin except for short periods, after injury, in some instances (Bernstein et al., 1985; Liesi et al., 1984), but not in all tested nerves (McCloon, 1986). In contrast, astrocytes of developing rat, like Schwann cells, were shown in primary cultures, to produce and deposit laminin into the extracellular matrix (Liesi et al., 1983).

It appeared that laminin expression is in correlation with growth (Hopkins et al., 1985; Liesi, 1985; Williams and Varon, 1985). Thus, while it is expressed continuously in adult fish optic nerve it is absent in mammalian adult optic nerves. It was therefore tempting to speculate that the absence of laminin along with other extracellular matrix components and cell adhesion molecules in the CNS of mammals is a cause for their poor ability to regenerate, and that this can be circumvented by external stimuli.

2.4. A regeneration-like response in a nonregenerative system can be triggered by diffusible substances originating from growing nerves

We have shown that soluble substances originating from regenerating fish optic nerves, when applied to injured adult rabbit optic nerves, cause manifestations of early events of regeneration in the latter (Schwartz et al., 1985). The response includes changes in retinal protein synthesis and in growth activity in vitro. Moreover, a similar effect is induced by media conditioned by newborn rabbit optic nerves (Hadani et al., 1984). We therefore proposed the existence of a correlation between ability of nerves to grow and the ability of their environment to provide appropriate factors termed by us as growth associated triggering factors (GATFs; Hadani et al., 1984). These results laid the foundation for our research. The goals which we set for ourselves during the first year of USMRDC support was (i) to determine the extent of the in situ morphological effect induced by these factors (i.e., by the conditioned media of the regenerating fish optic nerves or the newborn rabbit optic nerves, GATFs); (ii) to elucidate the possible biochemical changes induced in the nerves in addition to those already observed in

the retinae; (iii) to find out the primary target of the applied substances and thereby to find out their mode of action; and (iv) to get an insight to their possible biochemical nature.

The major findings that we have obtained towards these goals are summarized below.

2.4.1. Growth in GATFs-treated injured nerves

Morphological studies provided evidence that media conditioned by regenerating fish optic nerve (which from now on will be termed GATFs defined above in 2.3) cause alterations in the injured adult rabbit optic nerve manifested by the prolonged survival of the injured fibers and by the appearance of growth cones (in contrast to their absence in the non-treated nerve) (Lavie et al., 1987). The growth cones were embedded in astrocytic processes. It was therefore suggestive that the glial cells of the treated nerves are probably providing cues needed for axonal growth. This further suggested to us that GATFs include factors which can activate glial cells to acquire growth supportive characteristics.

2.4.2. GATFs include factors which activate glial cells

In an attempt to find out whether media conditioned by regenerating nerves have the capacity to activate glial cells, we analyzed the neural environment subsequent to the application of GATFs.

Adult rabbit optic nerves were removed and examined immunocytochemically using goat antibodies directed against mouse laminin. Intact nerves and two kinds of injured optic nerves were studied. The latter include nerves that had been crushed and then implanted with a silicone tube containing collagen soaked in fresh medium and nerves that, subsequent to crush, were implanted with a silicone tube containing collagen saturated with GATFs (Solomon et al., 1985). Medium conditioned by

regenerating fish optic nerves served as the source of GATFs. This preparation was shown to be free of laminin .

It appeared from our study that the environmental changes caused by GATFs include those which lead to accumulation of laminin (Zak et al., 1987), an extracellular matrix component known to play a role in support of neural growth and elongation (Williams and Varon, 1985). Without application of GATFs laminin immunoreactive sites are not present within the extracellular matrix of the injured adult rabbit optic nerve. The more widespread distribution of laminin resulting from the application of GATFs could be directly induced by the GATFs or could result from a cascade of events initiated by the GATFs. It could involve increased production or secretion of laminin or other metabolic changes which affect its accumulation.

Our preliminary results, as well as reports in the literature, reveal that, in intact fish optic nerve (a regenerative system), in contrast to rabbit optic nerve laminin immunoreactive sites are present throughout the nerve matrix mainly in tongues of connective tissue which project in, from the periphery (Liesi, 1985; Hopkins et al., 1985). The continuous presence of laminin in the fish, like other components needed for growth, may be associated with the general plasticity in this system (Bawnik et al., 1986; Quitschke and Schechter, 1984). It therefore appears that the absence of laminin in the matrix of the injured mammalian optic nerve may be a cause, partially circumventable by GATFs, for its poor inherent regenerative ability. Further studies have been carried out in the last year towards the identification of the active substances within the CM responsible for the glial activation; and identification of other components within the CM which may be related to growth.

2.4.3. GATFs include factors which inhibit maturation of oligodendrocytes

Recently it has been shown that mature oligodendrocytes are non-permissive to axonal growth (Schwab and Caroni, 1988). We have shown in the last year of our research under this support, that media conditioned by regenerating fish optic nerves unlike media conditioned by noninjured fish optic nerves, contain factor which can inhibit maturation of oligodendrocytes of rat brain and optic nerves, and possibly thereby contribute to maintain conditions permissive for growth (Cohen et al., 1989). The results are summarized in Section 5.2.

3. RATIONALE FOR THE APPROACH TAKEN IN THIS WORK

In response to injury the adult CNS may exhibit several responses by both the glia and the axons which are needed for regeneration. Nevertheless these neurons rarely accomplish this process with functional recovery.

It is reasonable to believe that either all the needed events are occurring but not at the appropriate time, therefore leading to an abortive process or that one of the key elements in the process are missing and therefore the other needed events do not occur.

Our strategy was to study a system which is endowed with a high regenerative capacity (i.e., the fish visual system), thereby to get an insight to the nature of the problem in the mammalian CNS.

In the visual system of the fish we observed that injury causes environmental alterations. Based on these results we wanted to examine whether the regeneration supportive environment of a regenerative nerve, i.e., goldfish optic nerve, can make a neuron of the mammalian CNS (non-regenerative system) to express regeneration-associated response, having

in mind that if successful, it would indicate that (i) a nonregenerative system is defective in its environmental factors; and (ii) environmental changes in a regenerative system are involved in processes that enable production of factors needed for a regeneration. To carry out such a study, we developed a unique procedure of a transorbital surgery (Solomon et al., 1985) which enabled the use of an adult rabbit's visual system as our experimental model for a nonregenerative system.

At the onset of this study, we transplanted an optic nerve from a carp, which had been conditioned to regenerate by a crush injury, into a transected optic nerve of an adult rabbit by suturing the nerves end to end. However, immunological rejection was noticed after two weeks. To avoid the rejection, we used a "wrap around" implant with a silicone tube which was internally coated with collagen and contained diffusible substances (i.e., conditioned media) derived from regenerating optic nerves of fish (either carp or goldfish). As a result of this application the rabbit optic nerve and retina showed features which are characteristic of regeneration (Schwartz et al., 1985). These results raised the question as to whether injured optic nerves of adult rabbit have any potential to provide triggering factors. Our result showed that these nerves, do not have the potential. Two possibilities therefore came to mind: either that intact nerves have this potential but lost it as a consequence of injury, or that intact nerves do not have the potential to provide the triggering molecules. In the latter case the potential could have been lost during maturation if produced during development. Our experimental approach of a synthetic "wrap around" implant provided the means to resolve this issue. We were able to show that media conditioned by newborn nerves have a similar activity, while media conditioned by intact or injured adult rabbit optic nerves showed minimal or no activity (Hadani

et al., 1984).

It therefore seems that the ability of the axonal environment to provide triggering factors correlates with its growth state (regenerating fish optic nerve and neonatal rabbit optic nerve have the highest activity). This correlation justifies the conclusion that inability of mammalian CNS to produce active factors or to produce them at the appropriate time may be a reason for their poor ability to regenerate.

In an attempt to find out the mechanism underlying the observed effect in the implanted injured rabbit optic nerves we observed that the glial cells surrounding the injured nerves are also affected. These observations suggested to us that the active conditioned media (derived from actively growing nerves) contain several factors, including neuron specific factor, apolipoproteins and factors which have the capability to activate glial cells. Such activation is manifested by the acquisition of growth features, including production and accumulation of laminin (an extracellular matrix component needed for growth otherwise absent in adult mammalian CNS) and appearance of cell adhesion molecules.

These observations invite the speculation that the glial cells surrounding injured axons of the mammalian CNS like the neurons have the intrinsic ability to develop into regeneration supportive cells if activated properly at the right time. In the absence of such activation these cells will develop into gliosis forming cells which are inhospitable to injured axons growth.

It has therefore been desirable to isolate the regeneration activators; to identify the various components within the conditioned media originating from growing nerves; and to find an optimal way for their administration into nonregenerating nerves. This may lead to a way for intervention to facilitate regrowth after CNS injury by the least

invasive method, by making use of the system's own components and modulating them appropriately.

4. TECHNIQUES

4.1. Surgical procedures

4.1.1. Carps

Carps (Cyprinus carpio, 800-1200 gr purchased from Tnuva, Israel) were anesthetised with 0.05% tricaine methansulfonate (Sigma, Israel). Both optic nerves were then crushed with forceps, taking care to injure the nerve only and leave the surrounding tissue intact. Eight days after the injury, the injured nerves were dissected out and immediately transferred into serum free media (DMEM, GIBCO) for 1.5 h of incubation at room temperature

4.1.2. Rabbits

Rabbits (albino, Weizmann Institute, bred in Israel) were anesthetized by (5 mg/kg) xylazine and ketamine (35 mg/kg) administered subcutaneously. The left optic nerve was then exposed using the transorbital surgical approach. The optic nerve was crushed (for 30 sec, with a hemostatic clamp) 4-6 mm distal to the eye globe, and a "wrap around" implant was immediately applied (Solomon et al., 1985). The "wrap around" implant consisted of a silicone tube (2 mm and 4 mm internal and external diameters, respectively; Burke, FRG), coated inside with collagen (Zyderm, type V), which was soaked for 24 h at 11°C in media containing the active diffusible substances (conditioning media) or fractions obtained after separations.

4.2. Preparation of conditioned media

The excised fish nerve segments were incubated for 1.5 h in serum free media. At the end of the incubation period the media (300 μ l medium/4 nerves) were collected and stored at -20°C.

4.3. Application of horseradish peroxidase

Rabbit optic nerves were crushed and wrapped around with silicone tubes containing media conditioned by optic nerves of neonatal rabbits (Solomon *et al.*, 1985; Schwartz *et al.*, 1985; Hadani *et al.*, 1984). At various stages after this surgical manipulation, horseradish peroxidase (HRP, type VI, Sigma) was applied to a new cut in the optic nerve, made proximal to the eye, between the optic disc and the primary crush injury. Application of HRP was accomplished by applying surgical sterilized oxidised cellulose (Ethicon) soaked in 30% HRP (in phosphate buffer, pH 7.4). Forty-eight hours after the HRP application, the rabbits were reanesthetized and perfused gravitationally with phosphate buffer (0.1 M pH 7.4) through the exposed carotid artery. The whole retinas were then removed and mounted on precoated gelatin slides. The retinal slides were preincubated for 6 hrs in solution containing diaminobenzidine (DAB, 50 mg/100 ml), CoCl_2 (0.0025%), nickel ammonium sulphate (0.0002%) and dimethylsulfoxide (1%). At the end of the incubation the slides were transferred for 30 min into freshly-made phosphate buffer solution containing DAB (50 mg/100 ml) and covered with permount.

Animals were perfused further with 2% glutaraldehyde in phosphate buffer and the nerves were then excised from the optic disc to the chiasm. The excised nerves were incubated in phosphate buffer containing 30% sucrose. Cryosections (30 μ m thickness) were taken and stained for HRP using DAB as the chromophore of choice.

4.4. Immunocytochemical analysis for laminin immunoreactive sites of nerve sections

Normal nerves and those that had been crushed 7 days previously were removed and immediately placed into 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4 for 4 h at 4°C and then transferred into 30% sucrose for overnight incubation at 4°C. Eight micron cryostat sections were placed on gelatinized 8-spot slides (Shandon Scientific) and treated as follows: Sections were exposed to PBS containing 1% BSA (for 3 h at room temperature or overnight at 4°C) and then with 0.15 M Tris-HCl, 0.1 M glycine pH 7.2 (45 min at room temperature) to block nonspecific binding of antibody and autofluorescence due to fixation. Serum of sheep containing antibodies against murine EHS sarcoma laminin (diluted 1:1000, gift of Dr. H. Kleinman, NIH) or PBS containing 0.2 mg/ml BSA were then applied for 2 h at room temperature. Sections were washed (4x, 10 min each) in PBS containing 0.2 mg/ml BSA and then exposed to fluorescein isothiocyanate-conjugated rabbit F(ab')₂ anti-goat IgG (Cappel Laboratories, PA, 20 µg/ml, also reacts with sheep IgG) diluted 1:100 for an additional 2 h at room temperature. The sections were mounted in Gelvatol 20-30 (Monsanto Corp., NY) in Tris-HCl pH 8.2. Fluorescence was examined with a Zeiss photomicroscope III using a 100 watt mercury lamp and neonfluor 25x objective. All fluorescent micrographs were taken using 1 min exposures on Kodak Tri-X film and were developed identically.

4.5. In vitro approach for laminin production

4.5.1. Treatment of C-6 cells

C-6 cells were grown in wells of microtiter plates in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). When the cells reached confluency the medium was changed to Waymouth's medium

supplemented with insulin (5 $\mu\text{g/ml}$), bovine serum albumin (BSA, free of fatty acids, 0.5 mg/ml) and antibiotics (streptomycin/penicillin, 100 $\mu\text{g/ml}$). The cells were kept in this medium for 24 h, followed by an additional 24 h period in the same medium supplemented with media conditioned by the regenerating fish optic nerves. The cells were then examined for thymidine incorporation and laminin appearance.

4.5.2. ELISA screening methods for surface antigens

Cells which were grown in microtiter plates, were centrifuged 3 min at 1000 \times g and then washed with phosphate buffered saline (PBS). Nonspecific sites were blocked by BSA for 30 min at room temperature. Following this incubation the supernatant was collected and antibodies at the appropriate dilutions were applied for 30 min. The cells were then washed with PBS and treated with the second antibodies conjugated to horseradish peroxidase (50 μl were added to each well for 1-3 h incubation at 37°C or overnight at 4°C). Cells were washed, and 100 μl of substrate was added to each well. Reaction was stopped by the addition of sodium azide (0.01 w/v) and citric acid (0.1 M). Absorption was recorded at 410 nm.

4.6. Two-dimensional gel electrophoresis

Samples for the first dimension of isoelectric focusing were prepared in denaturing solution containing 9.5 M urea, 2% NP-40 (nonidet P-40), 2% ampholines (1.6% pH 5.0 to 7.0, 0.4% pH 3.5 to 10.0), and 5% 2-mercaptoethanol. The second dimension was performed on a slab gel containing 15% acrylamide (O'Farrell, 1975). After electrophoresis in the second dimension, the gels were fixed and stained.

4.7. Immunoblot

The tested samples were separated by one- or two-dimensional gel electrophoresis (SDS-PAGE 15% polyacrylamide). On each slot of the gel, samples corresponding to 50 μ g of protein were applied. Following electrophoresis the proteins were blotted immediately onto nitrocellulose (0.45 μ m; Schleicher & Schuell, Germany) for 2 h, 200 mA in transfer buffer (15.6 mM Tris, 120 mM glycine pH 8.3; Anderson *et al.*, 1982). The blotted nitrocellulose was incubated overnight at room temperature in phosphate buffer containing 0.3% Tween-20 (polyoxyethylenesorbitan monolaurate, Sigma). The immunological reaction was carried out at room temperature, sequentially: incubation for 1 h with the primary antibodies diluted 1:100, wash, incubation for 1 h with horseradish peroxidase (HRP) conjugated to protein-A (diluted 1:1000) wash, and incubation with 4-chloro-1-naphthol until an optimal color had developed.

4.8. Solid phase radioimmunoassay

Wells of flexible microtiter plates were coated with CM derived from intact fish optic nerves and injured nerves excised 1 and 8 days after injury (250 ng protein in 50 μ l phosphate buffer). After overnight incubation the wells were washed with phosphate buffer containing 1% BSA and further incubated with anti-apo-A-I antibodies at various dilutions (1:200-1:50000). After 2 h of incubation the wells were washed, and further incubated with [125 I]goat anti-rabbit Ig (100000 cpm/well) for an additional 2 h. At the end of the last incubation the wells were washed, dried and counted.

4.9. Immunocytochemical localization at the electron microscope level

4.9.1. Fixation

For the studies at the light microscope level optic nerves were

dissected out, cleaned, and then incubated in paraformaldehyde (4%) in PBS pH 7.4 for 2 h at 4°C, after which they were transferred into sucrose solution (30%) for overnight incubation at 4°C. Cryosections (10 μ m thickness) were placed on gelatin coated slides and treated as follows: sections were incubated in PBS containing 1% BSA for 30 min at room temperature and then in solution containing 0.15 M Tris-HCl, 0.1 M glycine pH 7.2 for 20 min at room temperature in order to block nonspecific binding of antibody and background fluorescence due to fixation. Primary antibodies after affinity purification were incubated for 2 h at room temperature. Sections were washed (4x7 min each) in PBS and then exposed for 2 hrs to a fluorescein isothiocyanate conjugated goat anti-rabbit IgG (Cappel laboratories, Pennsylvania) followed by wash (4x7 min each). Fluorescence was examined with a Zeiss photomicroscope III using a 100W lamp and neofluar 16x objective. All fluorescent micrographs were taken using 1 min exposures on Kodak Tri-x-film and were developed identically.

4.9.2. Electron microscopy

Optic nerves were dissected out, cleaned and then kept in 2% glutaraldehyde 1% paraformaldehyde in cacodylate buffer 0.1 M. Following the fixation, the preparation was chopped in 1 mm segments, placed back in the fixative, and postfixed with osmium tetroxide, in cacodylate buffer. The preparation was then stained in uranylacetate (2%) in water for 30 seconds and dehydrated in ethanol in increasing concentrations of propylene oxide. Embedding was done in Epon. Polymerization was performed at 60°C for 3 days. Thick sections (1-3 μ m) were prepared using LKB II ultratom. These sections were stained with toluidine blue (1%). Thin sections (500-700 Å) were collected on copper grids. The grids were treated with H₂O₂ (10%) for 10 min at room temperature, then washed in

water. The washed grids were then incubated in a PBS solution containing ovalbumin (1%), Tween-20 (0.05%) for 15 min to block nonspecific binding. Grids were then treated with the first antibody (affinity purified rabbit anti-apo-A-I antibodies) for 2 h and then exposed to the second antibody which was goat anti-rabbit IgG tagged with colloidal gold particles (Janssen, Belgium), then washed again, and stained with uranylacetate saturated in ethanol (50%) and for 4 min in lead buffer. The sections were visualized in a Phillips 410 electron microscope.

4.10. Cultures of primary brain oligodendrocytes

The effect of CM on in vitro development of galc positive cells from 1 day postnatal rat brain. Brains from neonatal rats were dissected out and dissociated according to the procedure of McCarthy and deVellis. After 8 days in vitro, the oligodendrocytes were shaken off and seeded on poly-L-lysine coated coverslips (10^4 cells/well). Cultures were stained at 24, 48 and 72 h in vitro, for galc immunoreactivity by the indirect immunofluorescent technique using fluorescein conjugated goat anti-mouse IgM. For each time period in culture cells, nontreated cultures incubated in Sato were used as the 100% control. In experimental cultures, CM (12 μ g protein/ml) was added at the indicated time periods.

4.11. Assessment of heparin binding activity of apolipoproteins

4.11.1. Determination of affinity to protein

A column of heparin-Sepharose CL-6B (Pharmacia) gel (1 ml) was equilibrated with PBS (150 mM NaCl, 15 mM phosphate buffer pH 7.4) and operated at room temperature. Following sample application, the column was washed with at least 10 column volumes of the same buffer to remove nonbound material. Bound substances were then eluted by 1.5 M NaCl. Fractions of 1 ml were collected and tested by ELISA for their binding

capacity to apo-A-I antibodies.

4.11.2. Solid phase radioassay

The binding of [^3H]heparin (0.49 mCi/mg, DuPont-New England Nuclear Research Products) to apo-A-I was quantified by a solid-phase assay in 96-well plates (Nunc, Denmark). Apo-A-I (50 μl , 2.8 $\mu\text{g}/\text{ml}$) in PBS containing 0.02% NaN_3 was added to each well and incubated overnight at 37°C. The next day, bovine serum albumin (BSA, 200 μl , 2 mg/ml in PBS) was added to each well for 1.5 h incubation at 37°C. The plates were then drained and various concentrations of [^3H]heparin in 50 μl PBS in the absence or presence of a 100-fold excess of unlabeled heparin (porcine intestinal mucosa, grade II, Sigma) were added for an additional 2 h of incubation. Unbound [^3H]heparin was washed away with wash buffer (PBS containing Triton X-100, 0.1% v/v). Tritiated heparin was solubilized by incubation for 30 min at 55°C, followed by the addition of 0.05 N NaOH solution (200 μl) containing 1% SDS. Quantitative measurements were obtained using liquid scintillation counter (Beckman Instruments).

4.11.3. Reversibility of [^3H]heparin binding to apolipoprotein-A-I

Wells were precoated with apo-A-I (168 ng/ml, 6 pmol in PBS), washed and incubated for 2 h with [^3H]heparin (160000 CPM/well) in PBS. The plates were washed with wash buffer followed by the addition of PBS (100 μl) or PBS containing excess of unlabeled heparin (100-fold). At various periods thereafter, the amount of bound tritiated heparin was determined by solubilization and counting.

5. RESULTS

5.1. Purification of apolipoprotein-A-I from regenerating fish optic nerve and its characterization

Apolipoproteins form an important class of lipid-associating proteins which are involved in lipid metabolism and are essential for the biosynthesis and degradation of plasma lipoproteins (Mahley and Innerarity, 1983). Recent studies have provided evidence that accumulation of apo-E and apo-A-I are associated with regeneration of axons after injury in both the peripheral (PNS) and the central nervous system (CNS), respectively. Although apo-E is synthesized at similar rates in injured tissues of the CNS and PNS (Muller *et al.*, 1985), it accumulates only in the extracellular space of the PNS (Muller *et al.*, 1985). In the normal optic nerve of mammals, immunoreactive apo-E is found in astrocytes but not in any of the other nonneuronal cells (Boyles *et al.*, 1985), whereas in the peripheral nerve apo-E derives mainly from macrophages (Stoll and Muller, 1986).

In avians and fish, in which the plasma is devoid of apo-E, apo-A-I seems to be its functional counterpart, at least regarding the response to axonal injury (Dawson *et al.*, 1986; Harel *et al.*, 1989a). The synthesis but not the accumulation of apo-A-I increases in the degenerating optic nerve of avians, as does apo-E in the degenerating CNS nerves of mammals (Dawson *et al.*, 1986). Apo-A-I accumulates in the regenerating CNS of fish apo-A-I accumulates, as does apo-E in the PNS of mammals (Harel *et al.*, 1989a).

It has been suggested that the accumulation of apo-E in the extracellular space of the mammalian PNS is due to its ability to bind to heparin (Stoll and Muller, 1986). This may explain the failure of apo-E to accumulate in mammalian CNS, which is devoid of extracellular matrix

having heparin-like binding moieties (Carbonetto, 1987; Zak *et al.*, 1987). Plasma apo-A-I is generally considered as lacking heparin binding sites, (Quarfordt *et al.*, 1978), and therefore the possible mechanism underlying the accumulation of apo-A-I in fish CNS is addressed in this study.

We purified and sequenced apo-A-I derived from fish plasma and nerve and analyzed their heparin binding capability. The results suggest that fish apo-A-I, in contrast to apo-A-I derived from mammals, binds heparin, though with low affinity.

5.1.1. Isolation of apo-A-I from CM of regenerating fish optic nerves

Purification of apo-A-I to homogeneity was achieved in three steps. CM of injured fish optic nerves was applied to a size exclusion column (Superose-12, Pharmacia). (i) The apo-A-I content in the fractions was determined by ELISA using rabbit antibodies directed to fish plasma apo-A-I. Figure 1a illustrates that fractions 28-29 corresponding to a molecular weight 158000, had the highest binding to the antibodies, as has previously been shown (Harel *et al.*, 1989a). (ii) These fractions were pooled and were further applied to an anion exchange column (Mono-Q, Pharmacia). The collected fractions were examined as detailed above. As can be seen in Figure 1b, apo-A-I was recovered at a NaCl concentration of 0.2 M in fractions 13-15 from the Mono-Q column. (iii) These fractions were combined, lyophilized and were further applied to a reversed-phase column (PepRPC, Pharmacia). Apo-A-I was recovered in one fraction (Fig. 1c) at an acetonitrile concentration of 50%.

The purity of apo-A-I at each of these three steps of purification was tested by SDS-PAGE and is shown in Figure 2. Amino acid sequence analyses (Edman degradation using a 475-A pulsed liquid/gas

phase protein sequencer apparatus, Applied Biosystems, Inc., CA), revealed that 14 N-terminal amino acids in the nerve derived apo-A-I were identical to those of the plasma derived apo-A-I. A lower degree of homology was found between the apo-A-I of the fish and human plasma (Table I). The recovered apo-A-I represents about 60% of the expected apo-A-I. The amount of apo-A-I in CM was estimated by ELISA, using as a calibration curve the binding of purified plasma apo-A-I to known amounts of the specific antibodies. Accordingly, in 5.5 mg protein of CM, 11.8 μ g of apo-A-I were expected and only 6.5 μ g of apo-A-I were recovered (Table II).

5.1.2. Analysis of heparin binding capacity of apo-A-I

As can be seen in Figure 3, part of the apo-A-I in media conditioned by regenerating fish optic nerves was retained on a heparin-affinity column and could be eluted by salt (10-15%; Fig. 3). Application of purified apo-A-I derived from the nerve to the column resulted in a higher percentage of heparin binding, possibly due to the existence of additional non-apo-A-I heparin binding molecules within the crude CM, which were eliminated in the course of purification. Using the same affinity column, apo-E within CM of injured rat sciatic nerves (dissected out 8 days after injury) showed an apparently higher capacity for binding to heparin, almost 65% (Fig. 4).

In order to examine whether apo-A-I of the fish plasma can bind to a heparin column, we used the purified apo-A-I. Preliminary studies using the whole fish plasma resulted in no binding of apo-A-I immunoreactive molecules (Harel *et al.*, 1989b), presumably due to the presence of other heparin binding molecules within the plasma of higher affinity. Figure 5 shows, however, that apo-A-I purified from the fish plasma binds

heparin. For comparison, purified apo-A-I derived from human plasma was not retained on the heparin affinity column (Fig. 6), in agreement with previously reported results (Quarfordt et al., 1978). Our results therefore suggested that either apo-A-I from fish plasma and nerve binds heparin with low affinity, or that a copurified protein in these two preparations is responsible for the heparin binding.

To rule out the possibility that a contaminant, probably co-purified with apo-A-I, was responsible for the heparin binding we radioiodinated the purified apo-A-I from optic nerve CM and applied it to the heparin affinity column. The profile of binding was monitored by scintillation counting (Fig. 7a) and the bound fraction was analyzed by SDS-PAGE followed by autoradiography (Fig. 7b). The result revealed that a 28 kDa polypeptide was bound to the heparin-affinity column. This observation, therefore, left open the other possibility, namely, that apo-A-I did bind to heparin though with a low affinity.

This issue was addressed in the present study using two approaches. The first approach involved the application of the nonbound fraction (which was collected from the apo-A-I preparation after passage through the heparin affinity column) to a newly equilibrated column. The result showed that the nonbound fraction still contained heparin binding substances (data not shown). This procedure was repeated 4 times until the effluent of the columns was devoid of any further apo-A-I binding activity. The second approach involved analysis of the binding characteristics of apo-A-I to [3 H]heparin (Fig. 8a). The reversibility of the binding is shown in Fig. 8b. As can be seen, a 50% displacement by non-labeled heparin was achieved after 8 min of incubation. The result of these experiments analyzed by Scatchard plot revealed that apo-A-I binds to heparin with an average (obtained from 6 experiments) dissociation

constant of 2.8×10^{-6} M. Under the same experimental conditions, human apo-A-I failed to bind [3 H]heparin above the level of the nonspecific binding to the plastic.

Regeneration and degeneration have been correlated in mammals and in avians, and in fish with accumulation and lack of accumulation, respectively, of apolipoproteins (Muller et al., 1985; Dawson et al., 1986; Harel et al., 1989a; Skene and Shooter, 1983). Accumulation has been suggested to occur in the extracellular matrix, possibly via heparin binding domains (Stoll and Muller, 1986). Apo-E, indeed, has heparin binding sites and its binding to heparin displayed a dissociation constant of 6.2×10^{-7} M (Weisgraber et al., 1986). Apo-A-I in plasma of species in which it has been checked lacks such a binding capability, except for one instance in which direct evidence is lacking but in which the HDL fraction from humans showed heparin binding with low affinity. This HDL fraction contained apo-A-I (Weisgraber and Mahley, 1980).

In the present study, we show that apo-A-I free of any HDL particles, isolated from fish plasma and nerve, has an heparin ability (Figs. 3,5-8) with a dissociation constant of one order of magnitude lower than that of apo-E (2.8×10^{-6} M) (Fig. 8). The heparin binding ability of apo-A-I is significantly lower than that of laminin which was similarly tested (Skubitz et al., 1988). Under our experimental conditions, apo-A-I, isolated from human plasma, did not show any binding of [3 H]heparin above background (Fig. 6).

It therefore appears that fish apo-A-I behave differently from apo-A-I of other species with respect to heparin binding, resembling in this respect the mammalian apo-E, which is missing in the fish. This functional similarity might suggest also structural similarity of fish apo-A-I and mammalian apo-E. It is possible that the apo-A-I is related

to the evolutionary ancestor of the mammalian apo-E. Such a possibility was suggested recently in the chicken, with respect to other apolipoproteins (Sastry and Karathanasis, 1988). More direct proof in the fish will await the isolation and characterization of the gene encoding for apo-A-I. This work is currently in progress.

5.2. GATFs include factor(s) which block differentiation of oligodendrocytes from their progenitors

The inability of mammalian central nervous system (CNS) neurons to regenerate has been attributed to the inhospitability of their surrounding environment. Thus, for example, injury stimulates proliferation of astrocytes to form a scar tissue which impedes regeneration (Reier et al., 1983). More recently, it was suggested that the scar tissue is formed by type-1 astrocytes (Muller et al., 1986), possibly nonsupportive for regeneration not as a physical constraint but as a barrier of deficiency in molecules needed for growth support. Failure of axonal regeneration has also been attributed to the inhibitory effect of oligodendrocytes or their myelin products (Schwab and Caroni, 1988).

As was mentioned above in Section 2.4, we showed that application of conditioned media derived from regenerating fish optic nerves into injured adult rabbit optic nerves resulted in growth of axons (Schwartz et al., 1985).

We examined whether such CM also have an effect on oligodendrocytes, possibly by inhibiting or restraining their maturation, thereby preventing them from developing a nonpermissive environment for growth. Cultures of newborn brains and of injured adult rat optic nerves were used. Using the rat CNS model allowed us to use cell specific markers for oligodendrocytes in various stages along their cell lineage, which

have been well characterized in the rat such as A₂B₅, which labels oligodendrocyte type-2 astrocytes (O-2A) progenitors and type-2 astrocytes (Eisenbath et al., 1979; Raff, 1983), O-4, which labels immature oligodendrocytes (Sommer and Schachner, 1981), and adult O-2A progenitors (French-Constant and Raff, 1986), and galactocerebroside (galc), which labels mature oligodendrocytes (Raff, 1978).

In cultures of adult rat optic nerves, which had been crushed 3 days prior to their removal, the total number of multiprocessed O-4 positive cells was higher than in cultures of uninjured rat optic nerves (A. Cohen, T. Sivron, R. Duvdevani and M. Schwartz, in preparation). Figure 9a,b shows O-4 positive multiprocessed cells 96 h after seeding in cultures of rat optic nerves, which had been injured 3 days prior to their removal (Fig. 9a). In the presence of conditioned media derived from regenerating fish optic nerves (12 µg protein/ml) added to these cultures after 48 h in vitro (h in vitro), the number of O-4 positive cells that appeared at 96 h in vitro was much lower than in nontreated cultures kept in the defined medium (Bottenstein and Sato, 1979). Moreover, all the observed cells appeared as unprocessed and therefore seemed to remain as immature oligodendrocytes (Fig. 9c-g). These results were reproducible when the same experiment was repeated but with the use of galc antibodies, instead of O-4. Thus, in cultures treated with CM, only 42% of the cells developed into mature galc positive cells out of the total galc positive cells in the defined medium. These results suggested that the CM of the regenerating fish optic nerves contain factor(s) which can prevent differentiation of oligodendrocytes.

To examine possible mechanisms underlying the observed effect subsequent experiments were carried out using oligodendrocytes of neonatal brain. The use of newborn brain oligodendrocytes allows higher yield

of cells and performance of experiments, aiming at finding out at what stage in their cell lineage the effect is most pronounced.

Oligodendrocytes and their perinatal progenitors were obtained in these experiments by the methods of McCarthy and deVellis. The oligodendrocytes were seeded on poly-L-lysine coated coverslips. Abundant multi-processed galc positive cells were observed 24 h after seeding in Sato medium. When CM was added (12 μ g protein/ml) at the time of seeding, the number of galc positive cells developed at 24 h in vitro was significantly lower than in the control medium. Under the same experimental conditions, 1.2 μ g protein/ml CM of regenerating fish optic nerves did not have any effect. At 48 h in vitro further reduction was noticed (Fig. 10). Addition of regenerating CM at 24 h in vitro or 48 h in vitro resulted in a similar reduction in the number of differentiated galc positive cells, but only transiently (Fig. 10). The apparent transient effect might be attributed to the fact that cells which had already passed a certain stage of differentiation would continue to maturation, even in the presence of the CM. These results suggest that CM affect oligodendrocyte lineage at their early state, namely, at the O-2A progenitor level. This conclusion is further supported by comparison of CM and PDGF effects.

PDGF has recently been suggested as a factor which controls time and rate of production and differentiation of oligodendrocytes (Noble et al., 1988; Raff et al., 1988). We therefore examined whether the effect observed with CM could be reproduced by PDGF. Addition of PDGF to brain cultures at the time of seeding resulted in a reduction in the number of galc positive cells at 48 h in vitro. However, the effect was less marked than that observed with the CM (Fig. 11). Since PDGF has been shown as a mitogen of O-2A progenitors, the apparent lower number of galc

positive cells observed in PDGF-treated cultures could be a reflection of proliferation of the progenitors and thereby delaying differentiation towards oligodendrocytes.

In order to find out whether the observed effect of CM was similar to the mechanism by which PDGF works, we counted the number of progenitor cells stained with A₂B₅ antibodies in CM- and PDGF-treated cultures. As can be seen in Figure 11, there was a striking difference in the number of A₂B₅ in PDGF-treated cultures, as compared with their number in CM-treated cultures. Thus, in PDGF-treated cultures, as expected, there was an increase in the number of O-2A progenitors, stained with A₂B₅ antibodies (Fig. 11a,c,d). In contrast, in CM-treated cultures there was a reduction in the number of A₂B₅ positive cells (Fig. 11c,d). These results, thus, ruled out the possibility that the observed effect of the CM on the development of galc positive cells from their progenitors is mediated by PDGF-like activity or any other mitogens. This is further substantiated by the results illustrated in Figure 12, which show that addition of CM together with PDGF resulted in a reduction of galc positive cells, as would be expected from the application of CM only.

To establish a more quantitative assessment of the CM effect on oligodendrocytes, we used ELISA. Multiwell microtiter plates were used. In each well, 10³ cells were seeded. The level of galc immunoreactivity was determined by using mouse galc antibodies, followed by horseradish peroxidase conjugated with goat anti-mouse antibodies (HRP-GαM). Using such an assay, the effect of CM (Fig. 13) was similar to that obtained by immunofluorescence (Fig. 13, inset).

In this experiment, we also examined the effect of media conditioned by uninjured fish optic nerves, in comparison to that observed with media conditioned by regenerating nerves. As can be seen, media

conditioned by noninjured normal nerves had a negligible effect. Thus, the same number of galc positive cells appeared in cultures treated with CM of noninjured nerves as in the control of nontreated cultures. That the effect of the regenerating CM is selective to oligodendrocytes lineage was evident from the survival and differentiation of type-1 astrocytes in mixed brain cultures treated with the CM (Fig. 14).

We favor the interpretation that the CM of the regenerating fish optic nerves prevent differentiation of oligodendrocytes and is preferentially effective on their O-2A adult (Fig. 9) or perinatal (Figs. 10-14) progenitors. The observed effect of the fish regenerating CM seemed to be selective to oligodendrocytes, and is not a nonspecific toxic effect. This is evident (i) by the lack of inhibitory effect of the CM on other types of cells such as C-6 glioma, previously shown (Cohen and Schwartz, 1979) and on astrocytes in mixed glial primary cultures from newborn rat brains (Fig. 14); and (ii) by the fact that at the same protein concentration, media conditioned by uninjured nerves had a negligible effect.

The observed effect of media conditioned by regenerating nerves on differentiation of oligodendrocytes might imply (i) that successful regeneration in the fish CNS, at least in part, might be attributable to elevation in factors which can prevent differentiation of oligodendrocytes, otherwise a nonpermissive environment for growth; and (ii) that the apparent growth in injured rabbit optic nerves (Schwartz et al., 1985) treated with CM of regenerating fish optic nerves might be due to the in vivo effect of these components in modulating astrocytes (Zak et al., 1987; Cohen and Schwartz, 1989, and oligodendrocytes (present report) otherwise nonsupportive and nonpermissive for growth. Current efforts are therefore being made to isolate the factor(s) responsible for this novel activity.

5.3. Qualitative and quantitative analyses of the growth induced into the injured adult rabbit optic nerves

We showed at the beginning of our work under the support of the U.S. Army Medical Research and Development Command, that application of CM of regenerating fish optic nerves resulted in growth but to a limited extent (Lavie et al., 1987). Lately, we combined this treatment with a treatment modality which delays the posttraumatic degeneration of the mammalian optic nerves (Assia et al., 1989; Schwartz et al., 1987). Experiments were carried out by application of CM to almost complete transected adult rabbit optic nerves and a daily irradiation (5 mm, 35 mW) with a low energy He-Ne laser commencing immediately after injury.

In order to evaluate the extent of degeneration of the adult rabbit optic nerves which resulted from the injury and to provide a basis for evaluating the effects of the treatment used in this study, we first describe intact rabbit optic nerves and then transected (operated control) rabbit optic nerves.

5.3.1. Normal rabbit optic nerve

The normal rabbit optic nerve is composed of myelinated axons, grouped in fascicles and surrounded by astroglial cytoplasm (Fig. 15). The nerve is covered by a meningeal membrane. Counts of axons from examination of the sections with EM revealed that unmyelinated axons make up less than 2% of the total population (see also Vaney and Hughes, 1976) and that these unmyelinated axons are not fasciculated but are distributed sparsely throughout the nerve.

Stereological analysis indicates that 62.8% of the cross-sectional area of the nerve is occupied by myelinated axons, 37.1% is occupied by glial cytoplasm, most of which can be identified as astroglial by the presence

of glial filaments, and a negligible fraction of the total area is occupied by profiles of unmyelinated axons. Analysis of the diameter and myelin index of the axons in intact rabbit optic nerves is shown in Figure 1c and 1d. The myelinated axons have a mean myelin index of 0.725 ± 0.080 , indicating a substantial myelin sheath, and a mean diameter of $1.424 \pm 0.718 \mu\text{m}$.

5.3.2. Transected rabbit optic nerve (operated control)

A piece of nitrocellulose soaked in serum free medium was inserted in each of the operated control nerves. Nerves were examined at 2, 4, 6 and 8 weeks postoperatively. The nitrocellulose film was clearly identifiable at all postoperative times in all animals. The most proximal segment containing nitrocellulose was considered to be the site of injury.

At 2 weeks, at a site 2 mm distal to the injury, most of the axons showed signs of degeneration (Fig. 16a), although some apparently healthy myelinated and unmyelinated axons could still be seen (Fig. 16b). At 6 weeks postoperatively (Fig. 16c), the cross-sectional area of the nerve was largely composed of collagenous tissue and glial cytoplasm forming the glial scar, some degenerating axons were also seen, but no viable axons could be recognized. Thus, even those axons adjacent to the meningeal sheaths which might have escaped direct damage had nevertheless degenerated by this time. In a few cases in which the lesion was less extensive, there were regions where the axons showed the organization, density and thickness of myelin sheath characteristic of the intact noninjured nerves. These were considered to be spared axons. These nerves were omitted from the analysis.

A reconstruction of an operated control nerve 8 weeks postopera-

tively, is shown in Figure 17. The nerve contains no viable axons from a point 1.5 mm proximal to the site of injury (arrow). Apparently, intact axons are present between the optic disk and the area of the complete degeneration.

These results provide the basis for our evaluation of the effect of the combined treatment.

5.3.3. Transected rabbit optic nerves subjected to combined treatment

We examined transected nerves from 23 animals with postoperative survivals of 2-8 weeks, into which nitrocellulose soaked with CM was inserted, and which were then exposed to laser irradiation for 14 consecutive days, a treatment modality which delays posttraumatic Wallerian degeneration (Assia *et al.*, 1989). Unmyelinated axons were observed in all treated nerves distal to the injury. Two nerves were reconstructed and then used for a systematic examination; axons in another 8 nerves were counted to provide an estimate of numbers of viable axons.

Figure 18 shows a diagram of a cross section of one injured treated rabbit optic nerve 2 mm distal to the site of injury, 6 weeks postoperatively. Five percent of the cross section of the nerve is occupied by a compartment containing viable axons and some degenerating axons, 30% is occupied by nitrocellulose, and the rest is occupied by glial scar, degenerating axons and connective tissue, largely collagen.

The compartment of viable axons contains thinly myelinated and unmyelinated axons (Fig. 19). Most unmyelinated axons are embedded in astrocytic processes. In this compartment, the following other features were observed: (i) structures resembling growth cones embedded in astrocytic processes (Fig. 20a,b); (ii) axons in close association with non-neuronal cytoplasm, provisionally identified as oligodendrocytes by vir-

tue of moderately dense cytoplasm and absence of glial filaments (Fig. 20c); and (iii) occasional appearance of unmyelinated axons within preexisting myelin cylinders (Fig. 20d). The area of degeneration merges with the area of viable axons (Fig. 20e) but is composed entirely of degenerating axons and glial cytoplasm.

In some cases, adjacent to the dura in both treated and operated control nerves, axons were seen in association with cells which resemble Schwann cells. The myelin periodicity in these axons was similar to that of axons in a peripheral nerve and was 18% greater than that of myelin formed by central glial cells (Table III and Fig. 21). These axons were considered likely to be peripheral axons based on their relationship to these Schwann cells and the periodicity of their myelin lamellae, and were not included among the axons counted in this study.

The astrocytic nature of the processes surrounding many of the axons is indicated by the presence of glial filaments and confirmed by labeling with antibodies against glial fibrillar acidic protein (GFAP) (Figs. 22 and 23). Figure 23 shows 11 unmyelinated axons which are embedded in a GFAP labeled process extending from the cell body of an astrocyte. These observations indicate that the astrocytes do not form a barrier to newly growing axons under these conditions.

In order to provide a more quantitative estimate of the number of unmyelinated and thinly myelinated axons within the nerve distal to the injury, thin sections were cut, mounted on 200 mesh grids and photographed. In a representative thin section from a treated nerve 6 weeks postoperatively, 2850 unmyelinated and myelinated axons were counted in the compartment containing viable axons. Other axon counts from treated nerves distal to the lesion are shown in Tables III and VI. It should be noted that the number of axons counted in all these cases are uncorrected

and underestimate the number of unmyelinated and thinly myelinated axons present in the compartment, since the grid bars obscure 57% of the section.

Another treated nerve, 8 weeks postoperative, was reconstructed from thin sections taken at intervals along its length (Fig. 24). The compartment containing viable axons decreases in size distally but extends 1.5 mm distal (section 9) to the last section containing the nitrocellulose film (section 6). In this nerve, therefore, axons appear to have grown 6 mm distal to the site of injury and 1.5 mm distal to the nitrocellulose.

The treated nerve showed in Figure 24 was also analyzed at two cross section levels: one was taken 1.5 mm and the other 3.5 mm distal to the site of injury. Figure 25a is a diagram of a cross section taken 1.5 mm distal to the injury and shows the position of the nitrocellulose and the compartments of viable axons, the glial scar and the connective tissue. The compartment of viable axons was further analyzed separately for the number and distribution of unmyelinated (b), myelinated (c), and degenerating (d) axons, and myelin index (e) and diameter (f) of myelinated axons. Figure 25b shows that unmyelinated axons are distributed throughout the compartment. Figure 11e shows the distribution of the myelin indices. Most of the myelin indices were greater than the control index, indicating myelin sheaths substantially thinner than those in mature uninjured optic axons. Some axons with myelin sheaths close to control values are found in the central portion of the compartment. Figure 25f shows the mean diameter of myelinated axons. Most of the myelinated axons in this compartment have a larger diameter than that of mature intact myelinated axons.

Figure 26 shows the results of an analysis similar to that shown

in Figure 11, from a cross section taken 2 mm more distally. Unmyelinated axons were restricted mainly to one zone (Fig. 26b). The area of the compartment of viable axons (14.9%) is smaller than the area occupied by viable axons (30%) at the more proximal site and there are also fewer viable axons more distally (Table IV). Comparison of the myelinated axons in these two sections of the treated nerves and of an unoperated control nerve, with respect to their diameters and myelin indices, is shown in Tables V and VI, and in Figures 27 and 28. The myelinated axons in the treated nerve have significantly higher myelin indices than the control uninjured nerve, indicating thinner myelin sheaths in the treated nerve (Table V). The diameter of the thinly myelinated axons in the treated injured nerves was also significantly larger than in unoperated controls (Table VI, Figs. 27 and 28).

5.3.4. Origin of the unmyelinated and thinly myelinated axons

Axons traversing the lesion site could originate from retinal ganglion cells, either as collateral sprouts from spared axons or as regenerating sprouts from injured axons. These axons could also be peripheral axons, which were severed during the optic nerve transection and which have regenerated into injured optic nerves. Those unmyelinated axons which were associated with cells resembling Schwann cells were omitted from our analyses.

To determine whether the viable axons not associated with Schwann cells were retinal in origin, we made intraocular HRP injections in unoperated controls, operated controls and in animals subjected to the treatment. HRP labeled axons were seen distal to the site of the injury in treated nerves at both light (Fig. 29a) and electron microscopic (Fig. 29b-e) levels. HRP reaction product is seen in unmyelinated axons sur-

rounded by collagen (Fig. 29b-d), by glial cytoplasm (Fig. 29b,c) and ensheathed by myelin (Fig. 29e). Note that in the electron micrographs some axons were not labeled with HRP. This is presumably a reflection of the vesicular nature of the WGA-HRP transported in the anterograde direction (LaVail and LaVail, 1973), since similar patterns of HRP labeling were seen in unoperated control nerves injected with HRP (Fig. 29f). As a further control, animals subjected to the combined treatment but not injected with HRP were processed for HRP staining to test for endogenous peroxidase. No endogenous activity was detected in those nerves.

5.3.5. Quantitative analysis of axons in nerves at 2 to 8 weeks postoperatively in treated and operated control nerves

We counted viable axons in treated nerves from animals at postoperative periods of 2-8 weeks. Table VI summarizes the number of viable axons at a distance of 2 mm distal to the site of transection in one nerve at each survival period. For comparison, a similar analysis was carried out in operated control nerves. In the treated nerves 2 mm distal to the site of injury, a much larger number of unmyelinated axons was present at each time period examined, compared to operated control nerves. Moreover, while there is a sharp decrease in numbers of axons at this site in the operated control nerves, there is no obvious indication of a decrease in number of axons in the treated nerves at longer postoperative intervals. This suggests that the outgrowth supported by the treatment occurs early and is not transient.

6. CONCLUSIONS

6.1. The poor ability of axonal regeneration after injury in the central nervous system has been attributed to the inhospitability of their cellular environment. Accordingly, it has been suggested that both astrocytes and oligodendrocytes in the adult injured mammalian CNS are forming nonsupportive and nonpermissive environment. Application of CM originating from regenerating fish optic nerves has resulted in a growth across this apparently otherwise inhospitable environment. We have shown last year that one of the component within the CM responsible for the activation of the environment is a factor which causes an increase synthesis and accumulation of laminin, an extracellular matrix protein, needed for growth, otherwise absent in mammalian CNS (Zak et al., 1987; Cohen and Schwartz, 1989). Recently, we have identified an additional component which affects oligodendrocyte maturation by interfering with their development for O-2A progenitors (Cohen et al., 1989). In the present report, we provide evidence for the presence of such oligodendrocyte inhibitors in media conditioned by regenerating fish optic nerves and its possible mode of action.

6.2. We showed that in CM of regenerating fish optic nerves, similarly to regenerating mammalian peripheral nerves, there is an elevation, relative to noninjured nerves, in apolipoproteins (apo-A-I in fish and apo-E in mammals). Last year, we have purified to homogeneity the nerve derived apo-A-I of the fish, sequenced it and provided evidence that it behaves similarly to mammalian apo-E and rather than mammalian apo-A-I, having heparin binding activity.

6.3. We have further extended our in vivo studies of regeneration. We have carried out systematic qualitative and quantitative studies which

have allowed us to show that axons are traversing the site of injury and by 8 weeks extending up to 6 mm distal to the site of injury. We have also counted the number of axons and provided a series of evidences that the viable axons in treated injured nerves are newly growing axons rather than spared axons.

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Table I: NH₂-terminal amino acid sequence

Amino acid no.		1	5	10	15
<hr/>					
Fish nerve	28 kDa protein	H ₂ N-ASP-GLN-PRO-PRO-SER-GLN-LEU-GLU-HIS-LEU-LYS-SER-ALA-ALA-GLN			
Fish plasma	Apo-A-I	H ₂ N-ASP-GLN-PRO-PRO-SER-GLN-LEU-GLU-HIS-LEU-LYS-LYS-ALA-ALA-GLN			
Human plasma*	Apo-A-I	H ₂ N-ASP-GLU-PRO-PRO-GLN-SER-PRO-TRP-ASP-ARG-VAL-LYS-ASP-LEU-ALA-THR			

* The sequence was taken from Shackelford and Lebherz, 1983 (24).

Table II: Yield of apo-A-I isolated from media conditioned by regenerating fish optic nerves

Crude CM μ g protein	Recovered apo-A-I	Expected apo-A-I (crude CM)	Yield %
5500	6.5	11.8	59.3

This table summarizes the yield of apo-A-I purified from CM. The amount of apo-A-I at each step was determined by titrating binding to apo-A-I antibodies, using as a standard curve the binding of purified plasma apo-A-I in a range of 0.05-5 μ g/ml.

Table III: Comparison of periodicity of myelin lamellae in axons associated with CNS glial cells and axons associated with Schwann cells

Type of nerves	Mean \pm SD (\AA)	
	Schwann cells	CNS glia
Unoperated control	229.0 \pm 11.2	194.0 \pm 2.49
Treated nerve	238.75 \pm 8.73	201.43 \pm 10.39

Periodicity of myelin lamellae was measured by diffractometry. In unoperated controls, the myelin was measured within optic nerve (CNS glia) and within a peripheral nerve in the orbit (Schwann cells). In the treated nerve, myelin was measured in axons within the optic nerve which were associated with cells which resembled Schwann cells and those more nearly resembling oligodendroglia. Two unoperated control nerves and two operated treated nerves were analyzed. Each analysis consisted of 10-16 axons.

Table IV: Comparison of number of axons in treated nerves 8 weeks postoperatively, at two levels distal to the site of injury

Type of axon	1.5 mm distal to site of injury	3.5 mm distal to site of injury
Unmyelinated	1102	344
Myelinated	12966	8137
Total viable axons	14068	8481
Degenerating	1900	2116

The numbers shown were counted from montages of the area of viable axons, but not corrected for the area covered by grid bars.

Table V: Comparison of myelin index in unoperated control nerves and in treated nerves, 8 weeks postoperatively

Type of nerve	Number of axons	Myelin index (M \pm SD)
<hr/>		
Unoperated control	195	0.725 \pm 0.080
Treated, S-3	114	0.81 \pm 0.099
Treated, S-6	118	0.79 \pm 0.065

Myelin indices in unoperated control axons are significantly less than the myelin indices in treated myelinated axons ($P < 0.001$, Student's T-test, 2-tailed). Axons in the more distal section (S-6) have significantly smaller myelin indices than in the more proximal section (S-3). S-3 and S-6 refer to section numbers shown in Figure 10.

Table VI: Comparison of diameters of myelinated axons in unoperated controls and treated nerves, 8 weeks postoperatively

Type of nerve	Number of axons	Diameter (M±SD)
Unoperated controls	196	1.424±0.718
Treated, S-3	117	1.722±0.647
Treated, S-6	118	1.978±0.800

Diameter of myelinated axons in unoperated control optic nerves is significantly less than in treated nerves ($P < 0.001$, Student's T-test, 2-tailed). S-3, S-6: section numbers indicated in Figure 10.

Table VII: Comparison of numbers of viable axons in operated control and in treated nerves 2 mm from site of injury, at 2-8 weeks postoperatively

Postoperative period (weeks)	Number of axons					
	Operated control			Treated		
	Unmyelinated	Myelinated	Total	Unmyelinated	Myelinated	Total
2	1127	5797	6924	7972	14858	22830
4	613	14547	15160	2263	22417	24680
6	0	0	0	3614	10338	13952
8	0	127	127	1109	20387	21496

These numbers were taken from one representative animal at each postoperative survival period. They are not corrected for the area covered by grid bars.

FIGURE LEGENDS

Figure 1: A three-step purification of apo-A-I from fish CM. (a) Separation of CM on a size exclusion column. CM of injured fish optic nerves was applied to a Superose-12 column (Pharmacia). The column was preequilibrated with buffer containing 50 mM NaCl and 5 mM phosphate, pH 7.4. Elution was performed with the same buffer at a flow rate of 0.2 ml/min. Fractions of 0.4 ml were collected and tested by ELISA for their binding capacity to apo-A-I antibodies. (b) Separation on anion exchange column. Combined fractions (28-30) from the Superose-12 column were applied to a Mono-Q column (Pharmacia). The column was preequilibrated with 5 mM borate buffer, pH 8.0, and elution was done by a linear gradient of buffer containing 150 mM borate buffer and 0.3 M NaCl. Fractions of 1 ml were collected and tested by ELISA for their binding capacity to apo-A-I antibodies. (c) Separation by reversed-phase chromatography. Combined fractions (13-15) from Mono-Q were applied to PepRPC column (Pharmacia). Elution was done by a linear gradient of acetonitrile. Fractions of 1 ml were collected and tested for their binding capacity to apo-A-I antibodies. Fraction no. 19 contained apo-A-I immunoreactivity.

Figure 2: Analysis by SDS-PAGE of apo-A-I in the various steps of its purification from CM. (a) Crude CM; (b) fractions collected after chromatography on Superose-12; (c) fractions eluted from Mono-Q; (d) fraction no. 19 eluted from reversed-phase column; (e) molecular weight markers: 92500; 66200; 45000; 31000; 21500; 14400 Da (Bio-Rad).

Figure 3: Heparin binding of apo-A-I within media conditioned by regenerating fish optic nerves. Medium conditioned by regenerating fish optic

nerves (280 μ g) was applied to a heparin affinity column (1 ml bed volume). The column was washed with PBS (15 column volumes). Elution of bound material was carried out with 1.5 M NaCl in PBS. Fractions of 1 ml were collected and tested by ELISA for their binding to apo-A-I antibodies. Note that only a part of the apo-A-I immunoreactivity was recovered in the heparin-bound fraction.

Figure 4: Heparin binding of apo-E in media conditioned by regenerating rat sciatic nerves. Media conditioned by rat sciatic nerves (180 μ g), prepared 8 days after injury, was applied to a heparin affinity column (1 ml bed volume). After extensive washing, elution was carried out with 1.5 M NaCl in PBS. Each fraction was tested for its binding to antibodies directed against rat plasma apo-E. Note that most of the apo-E immunoreactivity was recovered in the heparin-bound fraction.

Figure 5: Heparin binding of purified apo-A-I derived from fish plasma. Apo-A-I obtained from fish plasma HDL, followed by gel filtration and reverse-phase columns, was applied (10 μ g protein) to heparin-Sepharose, as described in Figures 3 and 4. As can be seen, part of the apo-A-I immunoreactivity was retained on the heparin column and recovered by salt.

Figure 6: Heparin binding of apo-A-I derived from human plasma. Human plasma-derived apo-A-I (16 μ g), obtained after delipidation of human plasma HDL particles, followed by gel filtration, was applied to heparin-Sepharose column. Apo-A-I immunoreactivity was tested with antibodies specific to human apo-A-I. Note the lack of heparin-bound apo-A-I.

Figure 7: Heparin-bound [^{125}I]apo-A-I derived from fish CM. (a) [^{125}I]apo-A-I from CM was applied to heparin-Sepharose column. The non-bound fraction was collected and the bound material was eluted after extensive washing. (b) Both nonbound and bound fractions were applied separately to an SDS-polyacrylamide gel (12%). The figure shows an autoradiogram of the gel.

Figure 8: Binding assay of apo-A-I to [^3H]heparin. (A) Various amounts of [^3H]heparin were added to wells precoated with apo-A-I (50 μl , 2.8 $\mu\text{g}/\text{ml}$). After 2 h of incubation at 37°C, unbound [^3H]heparin was solubilized and counted in Lumax-Xylene scintillation liquid. The specific binding of [^3H]heparin to apo-A-I was determined by subtracting the amount of [^3H]heparin bound to apo-A-I in the presence of a 100-fold excess of unlabeled heparin. Each value represents the mean of 2 experiments (in which 4 determinations from each sample were made). (B) Reversibility of apo-A-I binding to apo-A-I. [^3H]heparin (160000 cpm/well) was incubated for 2 h in wells coated with 6 nmol of apo-A-I. One hundred μl of PBS or PBS containing a 100-fold excess of unlabeled heparin, were added to the wells and then incubated for various periods of time. The bound [^3H]heparin was solubilized and counted.

Figure 9: Effect of fish CM of regenerating fish optic nerves on adult O-4 positive cells in cultures of adult injured pc-3 rat optic nerves. Cells were prepared (Ffrench-Constant and Raff, 1986) from adult rat optic nerves that were crushed 3 days before excision and were seeded on poly-L-lysine coated coverslips in defined medium (Sato). Cultures were stained for O-4 immunoreactivity at 96 h in vitro by an indirect immunofluorescent method, which was carried out as follows: Cells were first

incubated with mouse anti-O-4 antibodies for 30 min, followed by 30 min incubation with fluorescein conjugated goat anti-mouse IgM. At the end of the second incubation, the cells were washed and fixed with cold methanol (-20°C) for 10 min. Pictures (a) and (b) show, respectively, fluorescent and phase micrographs of the cells, after 96 h in vitro in Sato without any treatment. (c), (e), (f) and (g) show O-4 positive cells in cultures which were treated with CM-R (12 μg protein/ml) for 48 h prior to the staining. Pictures (c) and (d) represent fluorescent and phase contrasts of the same cells. Pictures were taken from one experiment which was reproducible in 2 additional experiments.

Figure 10: The effect of CM of regenerating fish optic nerves on in vitro development of galc positive cells from 1 day postnatal rat brain. Brains from neonatal rats were dissected out and dissociated according to the procedure of McCarthy and deVellis. After 8 days in vitro, the oligodendrocytes were shaken off and seeded on poly-L-lysine coated coverslips (10^4 cells/well). Cultures were stained at 24, 48 and 72 h in vitro, for galc immunoreactivity by the indirect immunofluorescent technique using fluorescein conjugated goat anti-mouse IgM. For each time period in culture cells, nontreated cultures incubated in Sato were used as the 100% control. In experimental cultures, CM-R (12 μg protein/ml) was added at the indicated time periods. The numbers represent the percentage of galc positive cells in each treated culture relative to those in the nontreated control Sato culture at the same time. In each case, the concrete number of galc positive cells on the entire coverslips was counted. In the control culture (Sato), the number of galc positive cells remained relatively constant throughout the experiment (24-72 h) and was

about 700-800 cells. This experiment was repeated twice and gave qualitatively the same results (N.D. - not done).

Figure 11: Comparison between effects of PDGF and CM of regenerating fish optic nerves on neonatal rat brain oligodendrocytes. Cultures of oligodendrocytes from neonatal rat brains were obtained as in Figure 2. In all cultures, an identical number of cells were seeded. At 48 h in vitro, cultures were stained for either galc or A₂B₅ positive cells. In treated cultures, either PDGF (5 ng/ml, Sigma) or CM-R (12 µg protein/ml) were added at the time of seeding. As control, we used cultures kept in defined medium (Sato) which were not treated. Fluorescent micrographs show cells stained with mouse A₂B₅ monoclonal antibodies in control cultures (b), cultures treated with PDGF (a), or cultures treated with CM-R (d). The bar graph in (c) shows the concrete counted number of A₂B₅ or galc positive cells in the coverslips of each treatment (space bars = 10 µm).

Figure 12: The combined effect of CM fish optic nerves and PDGF on development of galc positive cells. Cultures of brain oligodendrocytes were prepared as in Figures 2 and 3. PDGF (5 ng/ml) or PDGF together with CM-R (5 ng/ml and 12 µg/ml, respectively) were added 24 h after seeding. At the indicated subsequent times (24, 48, 96 h), cultures were stained for galc positive cells. The numbers represent the concrete numbers of galc positive cells per coverslip.

Figure 13: Comparison between the effect of CM of regenerating and intact fish optic nerves on development of positive cells in cultures of rat brain oligodendrocytes. Cultures of oligodendrocytes were prepared

as above, and seeded in multiwell (10^3 cells/microtiter plate). After 24 h in vitro, CM-R or CM-N were added at the indicated concentrations. Forty-eight hours later, the cells were examined by incubating first with galc antibodies at 37°C for 30 min, followed by horseradish peroxidase conjugated to goat anti-mouse antibodies (HRP-GαM, Bio-Makor, Israel), for additional 30 min at 37°C. Determination of the amount of bound antibodies was obtained by washing cells and adding 100 μl of substrate (2,2'-azino-di(3-ethylbenzthiazoline sulphate) (Sigma) to each well. Absorption was recorded in Titertech Multiskan MMC at 405 nm with a reference wavelength of 630 nm. Each bar represents an average (\pm SD) of 3 wells. The inset for comparison shows results obtained by counting the number of galc positive cells per coverslip in cultures treated with either CM-R (12 μg/ml) or CM-N (12 μg/ml) added at 24 h in vitro and examined 48 h later.

Figure 14: CM of regenerating fish optic nerves affect selectively oligodendrocytes in mixed newborn rat cultures of astrocytes and oligodendrocytes. Mixed glial cells were dissociated from newborn rat hemispheres, as described in Figure 2, and immediately were seeded on PLL coated coverslips (10^4 cell/coverslip). These cells were kept for 6 days in vitro in DMEM supplemented with 5% FCS, that was changed every 2 days. At day 6, the medium was changed into defined medium (Sato) and supplemented with CM-R (10 μg/ml) or CM-N (10 μg/ml). At 72 h in vitro and 96 h in vitro, the cells were double labeled with mouse monoclonal antibodies against galc and rabbit antibodies GFAP, followed by specific rhodamine conjugated goat anti-mouse IgG₃ and fluorescein conjugated goat anti-rabbit, respectively. Fluorescent micrograph (a) shows cells stained with

anti-GFAP and unaffected by CM-R treatment, after 72 h in vitro. (b) shows the concrete counted number of galc positive cells in the coverslips of each treatment, at 72 h in vitro. The results represent mean \pm SD of 2 coverslips. (c) and (d) are phase micrographs of typical mixed glial cells of untreated (c) and of CM-R-treated cultures (d). Note the monolayer cells under the CM-R-sensitive cells (d) remained unaffected, whereas the CM-R-sensitive cells are forming colonies and are stained with A₂B₅ in the control nontreated cultures (data not shown). These cells represent the O-2A progenitors that are stimulated to proliferate by the type-1 astrocyte monolayer underneath.

Figure 15: Electron micrographs of a cross section through optic nerve of a normal adult rabbit. (a) Axons are organized in fascicles and surrounded by processes of glial (gl) cytoplasm. (b) Astrocytes (As) are identified by abundant glial filaments and oligodendrocytes (ol) are recognized by dense nucleus and cytoplasm, absence of filaments and few microtubules. (c) and (d) show distribution of axon diameters and myelin indices, respectively, of this intact adult rabbit optic nerve. (a) 2800x; (b) 3400x.

Figure 16: Electron micrographs of operated nontreated control adult rabbit optic nerve, 2 mm distal to the site of the injury. (a) and (b) were taken from nerves 2 weeks postoperatively. (a) Most of the axons appear to be degenerating ones (dAx). Examples of apparently healthy myelinated axons (mAx) and unmyelinated axons (Ax) are shown in (b). (c) was taken from a nerve 6 weeks postoperatively. Most of the area was occupied by a glial scar (gls) which contained degenerating axons. (a) 2245x; (b) 2245x; (c) 13455x.

Figure 17: Reconstruction of an operated control adult rabbit optic nerve, 8 weeks postoperatively. Sections 2-9 are taken at 0.5 mm intervals. Sections were examined in the EM and compartments containing viable axons (Ax+mAx), glial scar and degenerating axons (Gls+dAx), glial scar (Gls), connective tissue (CT), dura matter (D), and nitrocellulose (NC) were identified. Note that the last section in which viable axons were present (section 4) is 1.5 mm proximal to the site of injury (section 8) in which nitrocellulose can be recognized. Arrow indicates site of injury.

Figure 18: A diagram of a cross section of a treated injured rabbit optic nerve, 6 weeks postoperatively. The cross section was taken 2 mm distal to the site of the injury. Compartments are designated as in Figure 3. The area occupied by viable axons in this section represents 5% of the cross section of the nerve.

Figure 19: Electron micrographs showing characteristics of a treated injured adult rabbit optic nerve 2 mm distal to the site of injury, 6 weeks postoperatively. Micrographs were taken from the compartment labeled by Ax+mAx in Figure 4. (a) shows an unmyelinated axon (Ax) enwrapped by glial cytoplasm (gl), surrounded by collagen (Col). (b) shows bundles of unmyelinated axons embedded in astrocytic processes (Ap) in a collagenous environment. (c) shows a bundle of unmyelinated axons and a myelinated axon (mAx) embedded in astrocytes (As). (d) and (e) show areas with high density of unmyelinated axons and of myelinated axons, respectively. (a) 12075x; (b) 7525x; (c) 15925x; (d,e) 6580x.

Figure 20: Some of the characteristics of axons in treated injured adult rabbit optic nerve. Micrographs (a-d) were taken from the same area from which the micrographs shown in Figure 5 were taken. (a) and (b) show growth cones (gc) surrounded by astrocytic processes (Ap). The growth cone in (a) is in continuity with an unmyelinated axon containing parallel microtubules. (c) shows axons ensheathed by dense cytoplasm indicating an oligodendrocytes (arrow). Micrograph (d) shows a rare example of growth within preexisting myelin cylinder. Note that in (d) the preexisting cylinder is also occupied by an astrocyte (As). Micrograph (e) shows an area of a glial scar (gls) containing degenerating axons (dAx) and phagocytes (phc). (a) 16380x; (b,c) 12420x; (d) 7740x; (e) 6580x.

Figure 21: Comparison between myelinated axons associated with Schwann cells and those associated with putative glial cells. The micrographs shown in this figure were taken from treated nerves 6 weeks postoperatively. (a) was taken from the area near the dura adjacent to but not included in the compartment of viable axons. We considered these axons to be peripheral axons myelinated by Schwann cells (Sc). (b) shows a region within the compartment of the viable axons containing astrocytes (As). Micrographs (c) and (d) show an enlargement of the myelin sheath of the axons shown in (a) and (b), respectively. Note that the periodicity of the lamellae in (b) is greater than in (d). (a,b) 10080x; (c,d) 109375x.

Figure 22: Postembedding immunocytochemistry using antibodies against GFAP. Sections were taken from a treated nerve 6 weeks postoperatively. The sections were taken 1.5 mm distal to the site of the injury. GFAP positive labeling is indicated by dense particles. Unmyelinated axons

(Ax) are embedded within GFAP labeled astrocytic processes (As). The bundle is surrounded by collagen (Col) which is unlabeled. Note that phagosomes (Ph) are also unlabeled. (a) 14490x (b) 22425x

Figure 23: Postembedding immunocytochemistry using GFAP antibodies. This picture shows 11 unmyelinated axons (Ax) embedded within GFAP-positive astrocytic processes. The astrocytic process (arrows) extends from the cell body (As), the nucleus (N) of which can be seen. The astrocyte and the embedded axons are surrounded by connective tissue (CT). 13975x.

Figure 24: Reconstruction of treated optic nerve 8 weeks postoperatively. The reconstruction was prepared from examination of a series of cross sections. Analysis and abbreviations, as in Figure 3. The first two sections were taken at a distance of 2 mm from each other and the remaining were taken at 0.5 mm intervals. Sections 2 to 6 contain nitrocellulose. Sections 1 to 9 show areas in which viable axons can be identified. The area containing viable axons decreases distally.

Figure 25: Quantitative analysis of axons in the viable compartment of treated injured adult rabbit optic nerve, 1.5 mm distal to the injury. (a) diagram of section 3. About 30% is occupied by viable axons, including myelinated (mAx) and unmyelinated (Ax) axons. A thin section from the region indicated by Ax+mAx was cut and placed on a 50 mesh grid, photographed and a montage was constructed. The montage was divided into squares of 50x50 μ m. Each square was analyzed for content of unmyelinated (b), myelinated (c) and degenerating axons (d). In each diagram shaded squares represent areas with values higher than the average. The

myelin indices of 20 axons in each square were measured (e); shaded squares indicate the presence of axons with a mean myelin index greater than the average plus the SD. The myelin index was greater than normal in most regions. The mean diameter of axons in each square is shown in (f).

Figure 26: Analysis of axons in the compartment of viable axons at a level of 3.5 mm distal to the injury in treated injured adult rabbit optic nerve (Fig. 24, section 6). Analyses are identical to those in Figure 11. The compartment of the viable axons occupies 14.9% of the cross section area.

Figure 27: Comparison between myelinated axons in unoperated control nerves and treated injured nerve. All axons in these photomicrographs were taken from subdural portions of the cross section of the nerve. (a) normal unoperated control nerve at a level of 5 mm distal to the globe. Axons are myelinated and densely packed. (b) treated nerve 8 weeks postoperatively, 1.5 mm distal to the site of injury. Axons are sparsely distributed, with large diameter and thinly myelinated. (c) 3.5 mm distal to the site of injury. Axons have thicker myelin sheaths than in (b), but still higher than in (a), and they are more densely packed than in (b). (a,b,c) 5040x.

Figure 28: Distribution of diameter and myelin indices of axons in Figure 27b,c.

Figure 29: HRP-labeled axons in treated injured and control adult rabbit optic nerves. Forty-eight hours before sacrifice, WGA-HRP was injected intraocularly. (a) light microscopic micrograph of HRP labeled fibers of

treated nerves (arrows indicate growing tips). (b-e) electron micrographs of axons in treated nerves. HRP containing vesicles (indicated by arrows) are seen in both unmyelinated axons (Ax) (b-d) and in myelinated axons (mAx) (e). (b) and (c) show examples of HRP-labeled unmyelinated axons embedded in astrocytic processes (Ap) and surrounded by collagen (Col). For comparison, HRP in myelinated axons of unoperated control nerve is shown in (f). (a) 1380x; (b,c) 17290x; (d) 16380x; (e,f) 17745x.

FIGURE 1

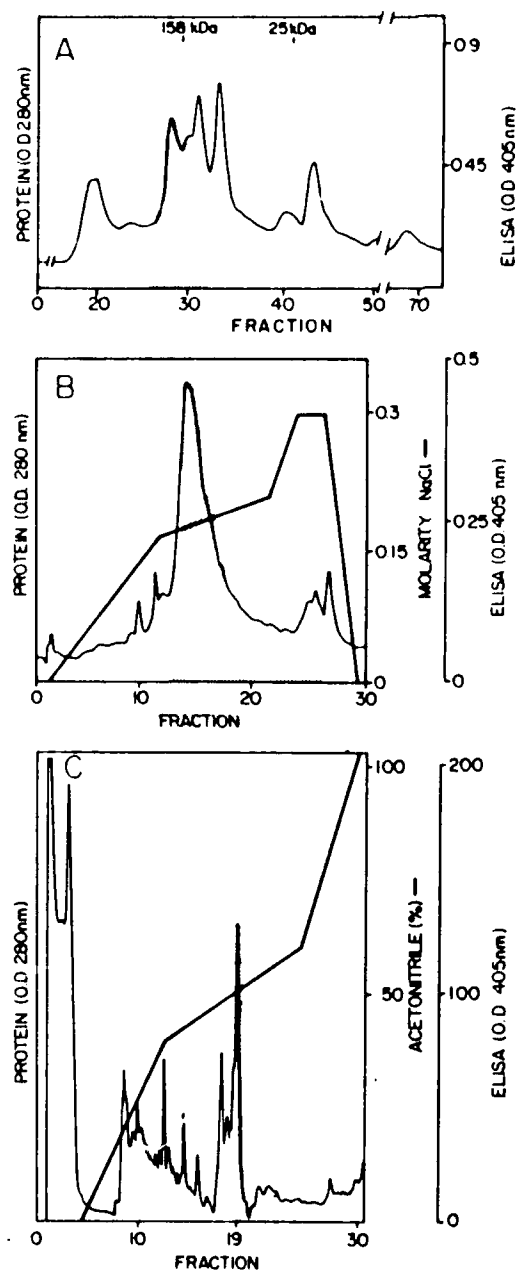


FIGURE 2

Steps in purification of apo A-1,
analyzed by SDS-PAGE.

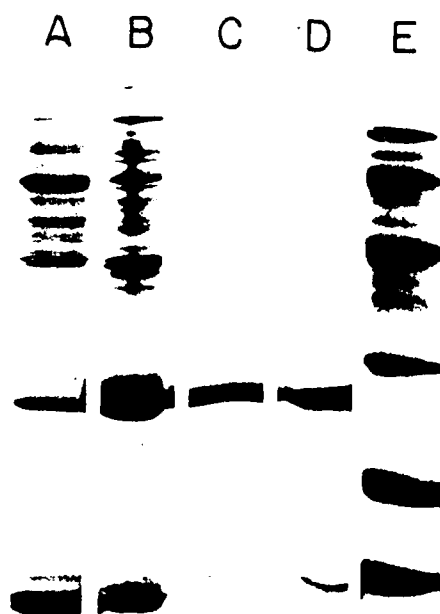


FIGURE 3

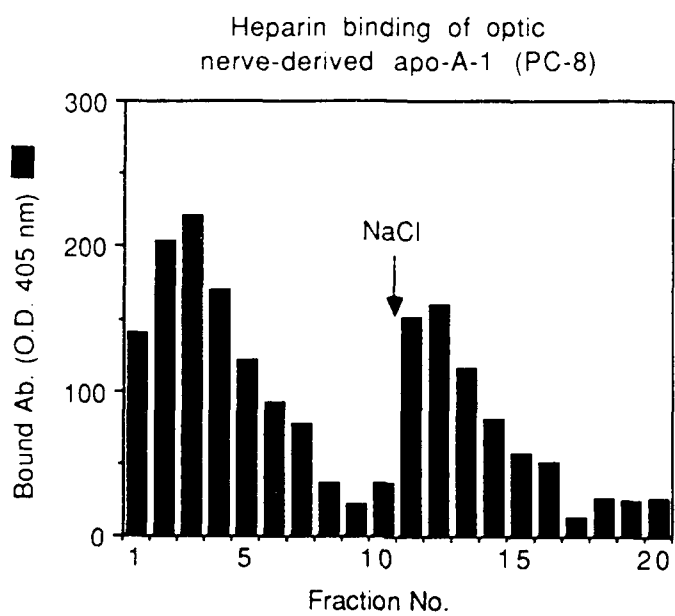


FIGURE 4

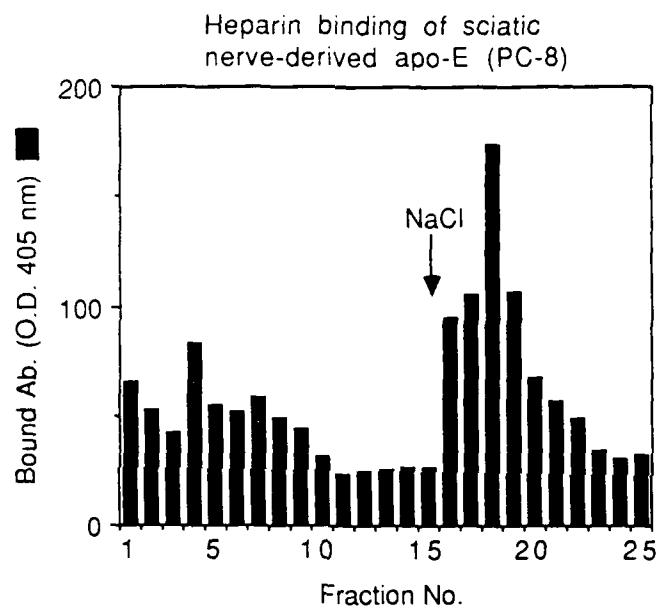


FIGURE 5

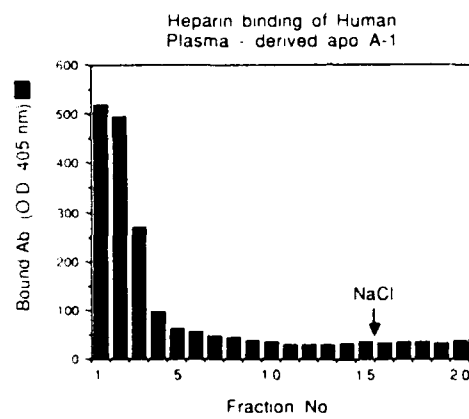
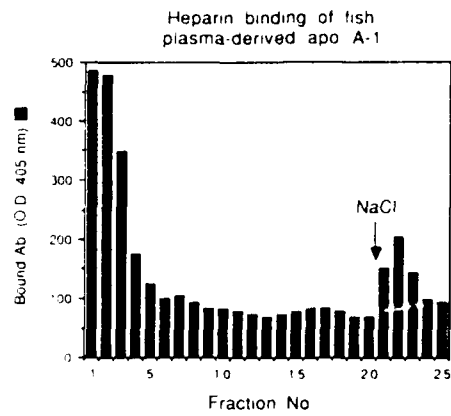


FIGURE 6

FIGURE 7

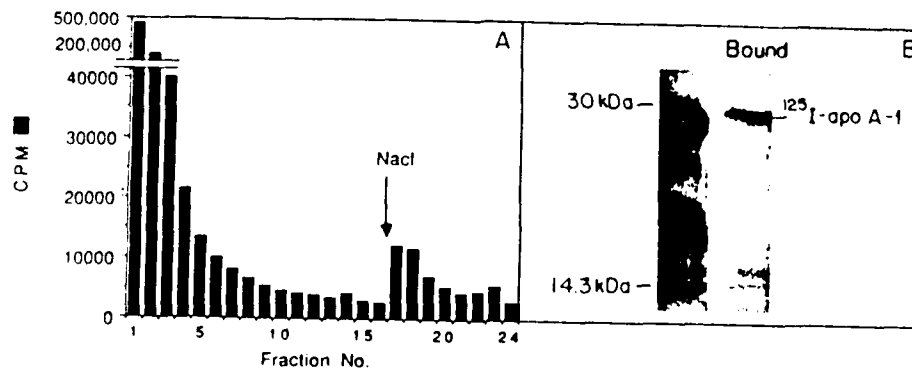


FIGURE 8

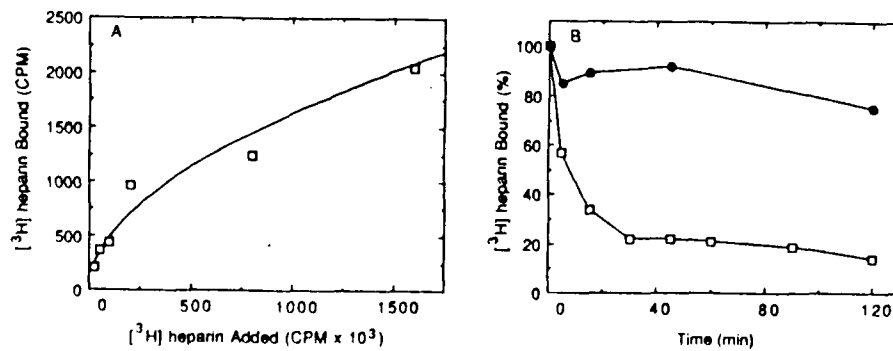


FIGURE 9

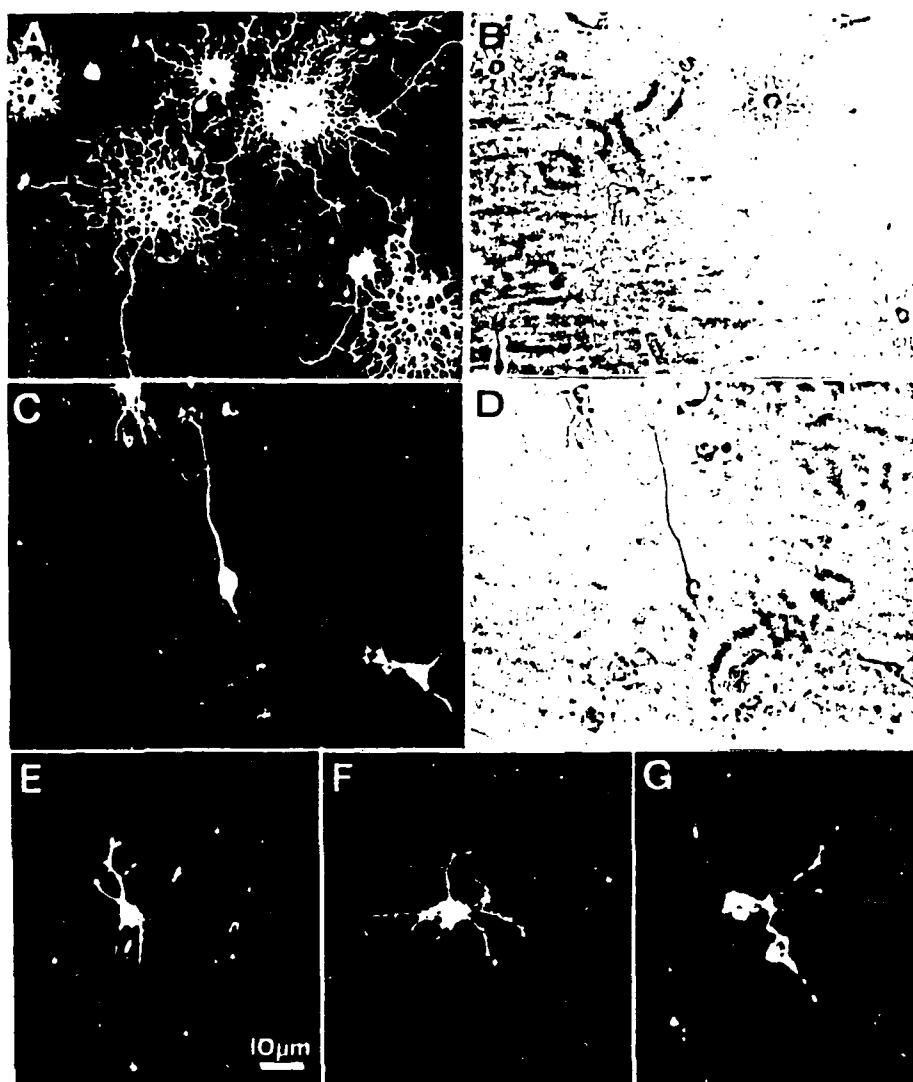
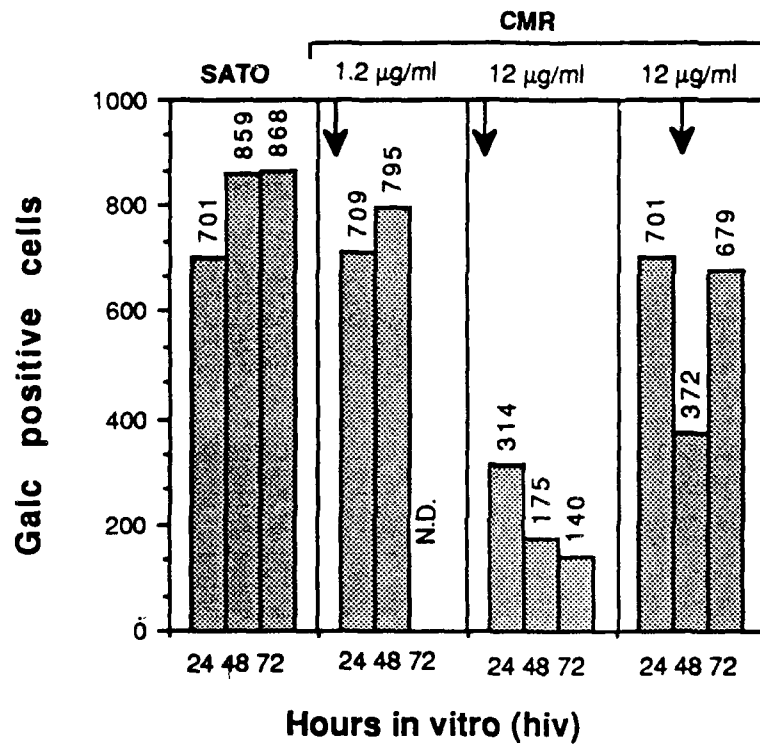


FIGURE 10



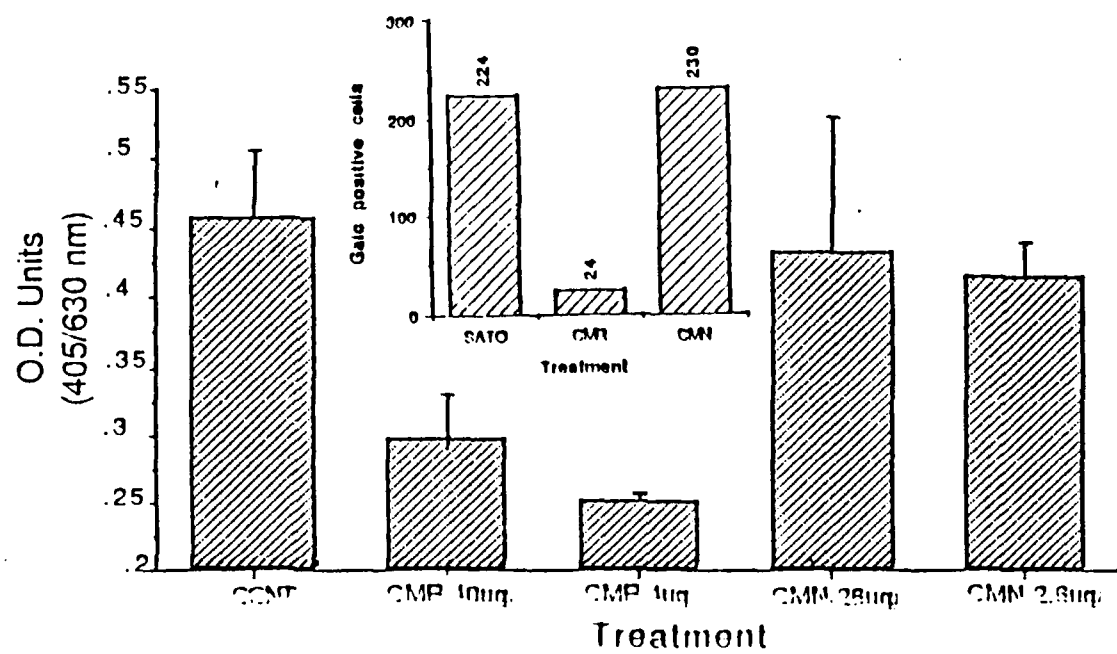


FIGURE 11

FIGURE 12

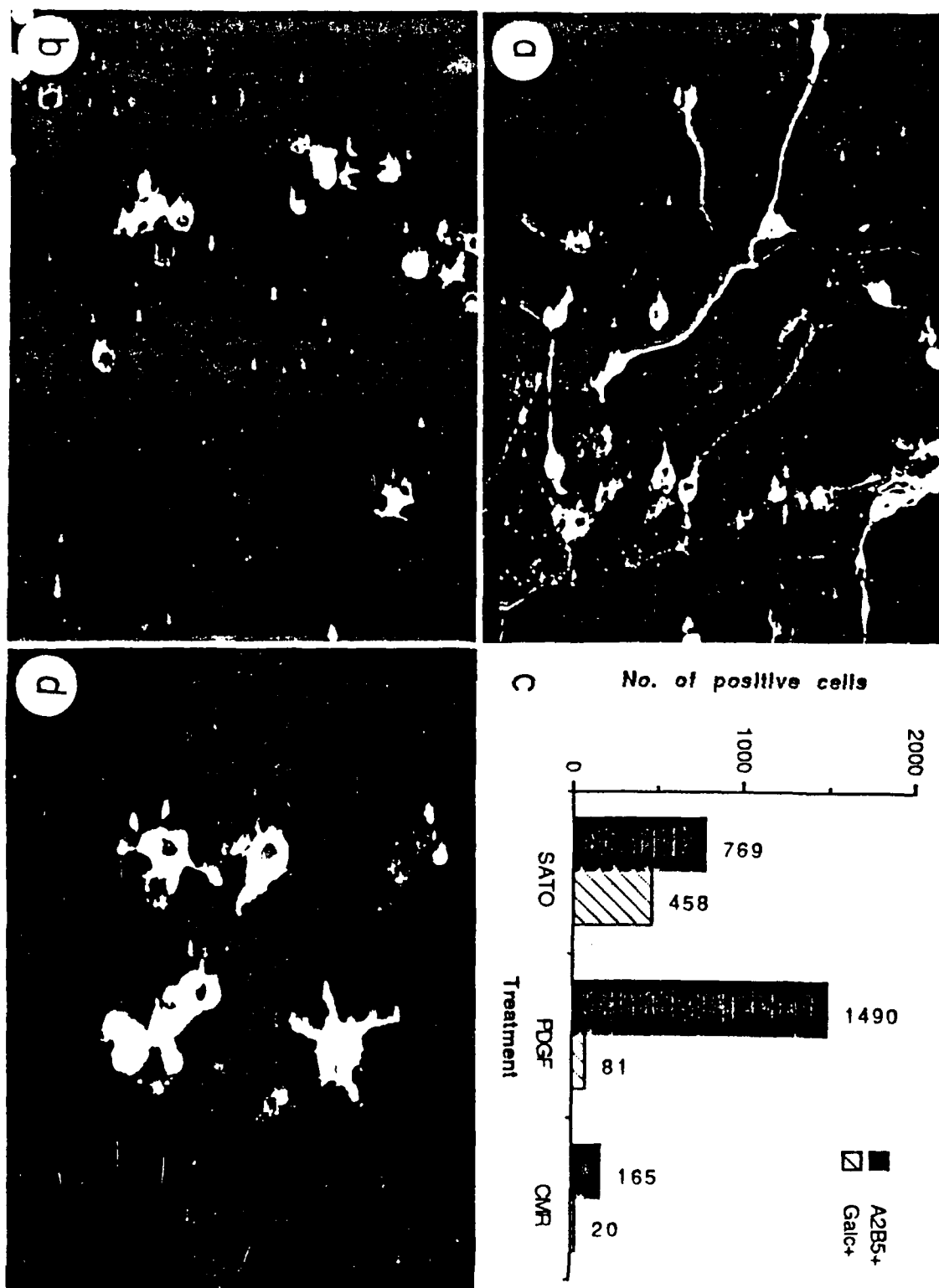


FIGURE 13

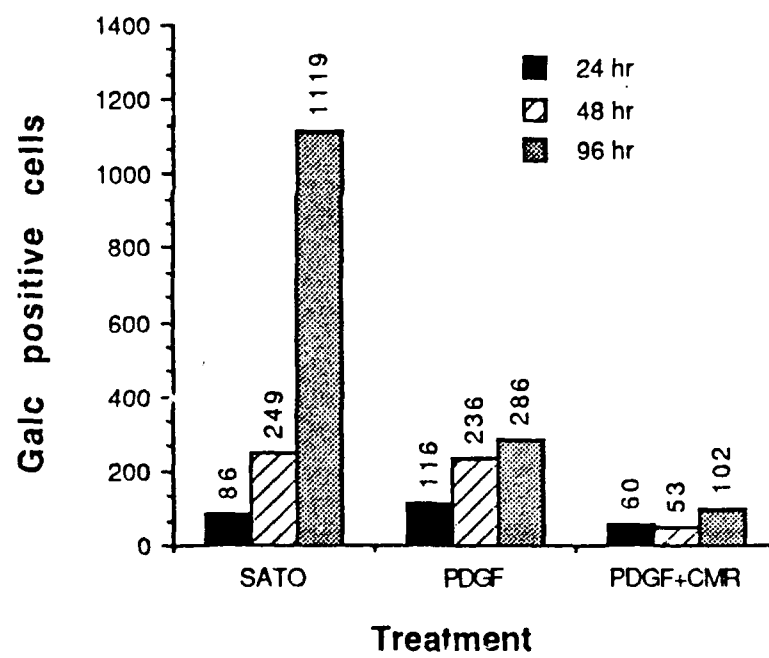


FIGURE 14

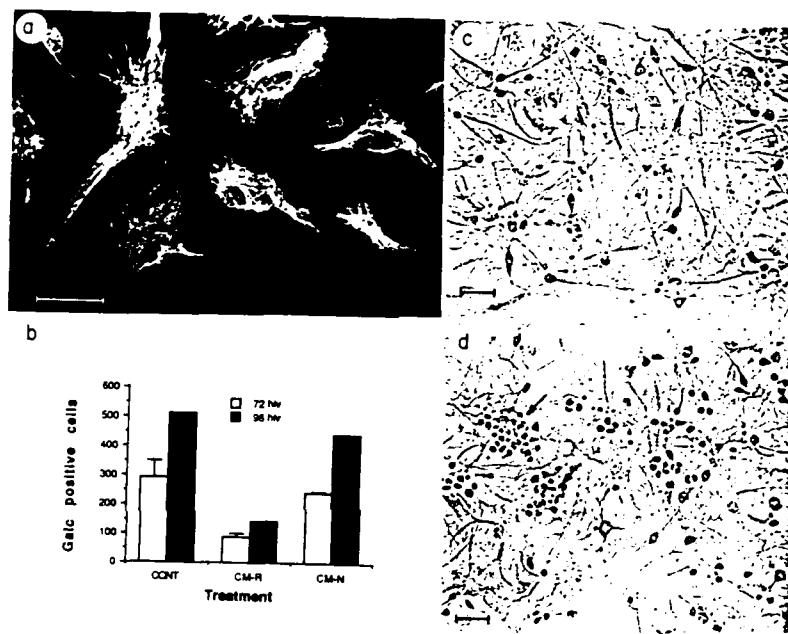


FIGURE 15

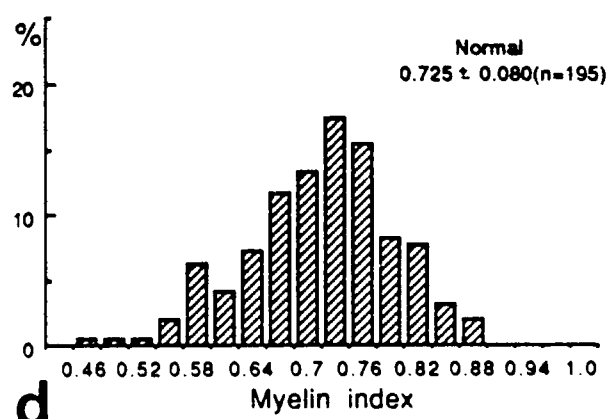
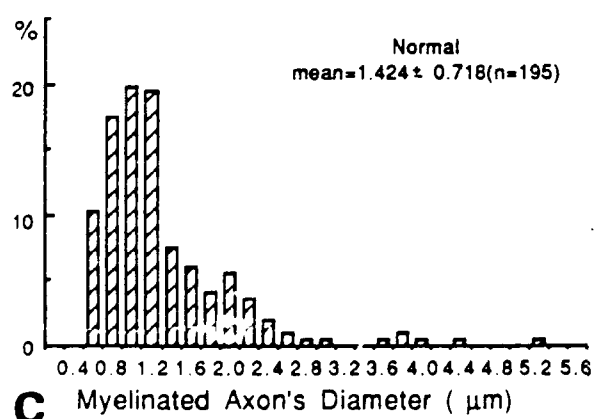
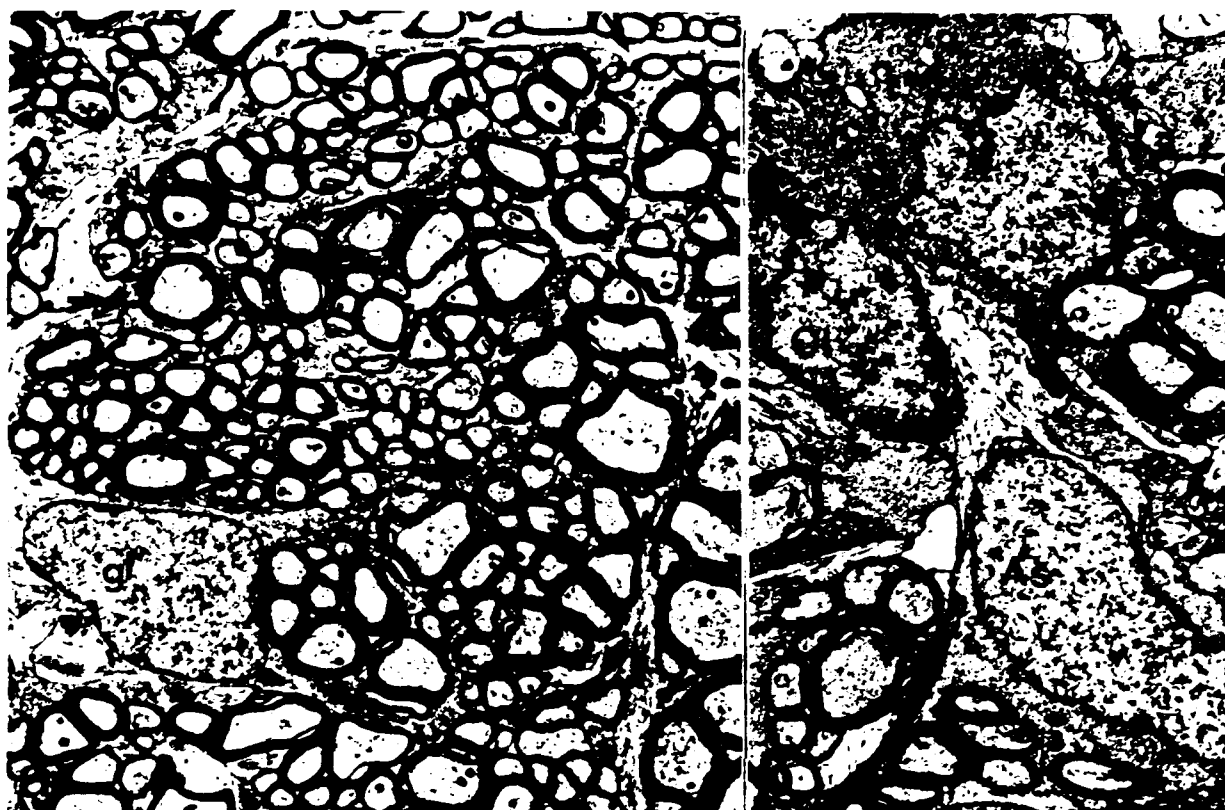


FIGURE 16





FIGURE 17

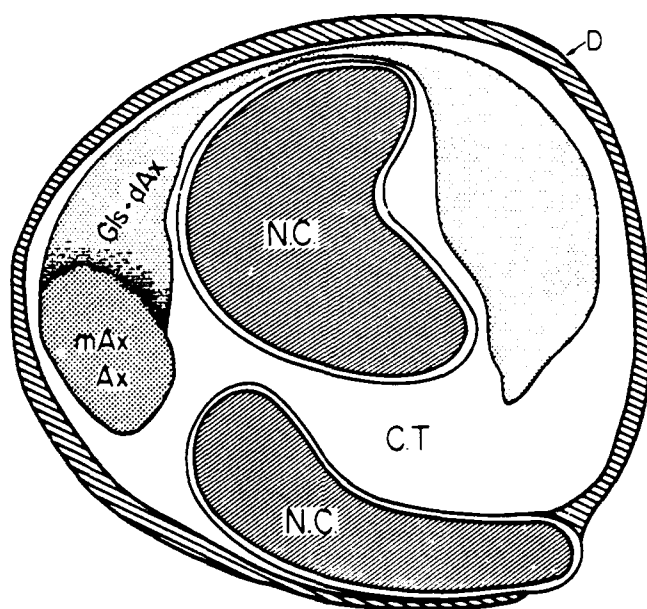


FIGURE 18

FIGURE 19

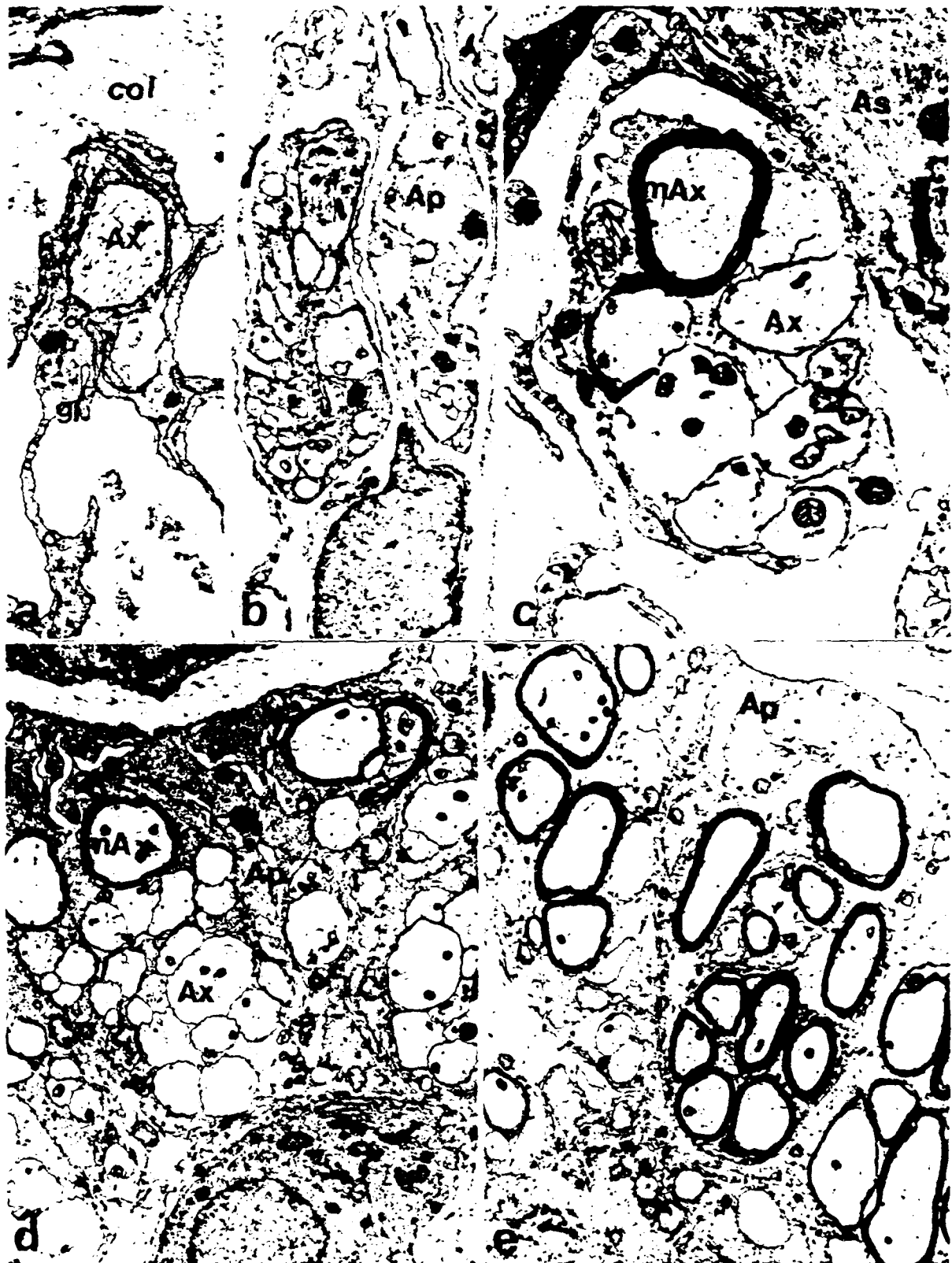


FIGURE 20

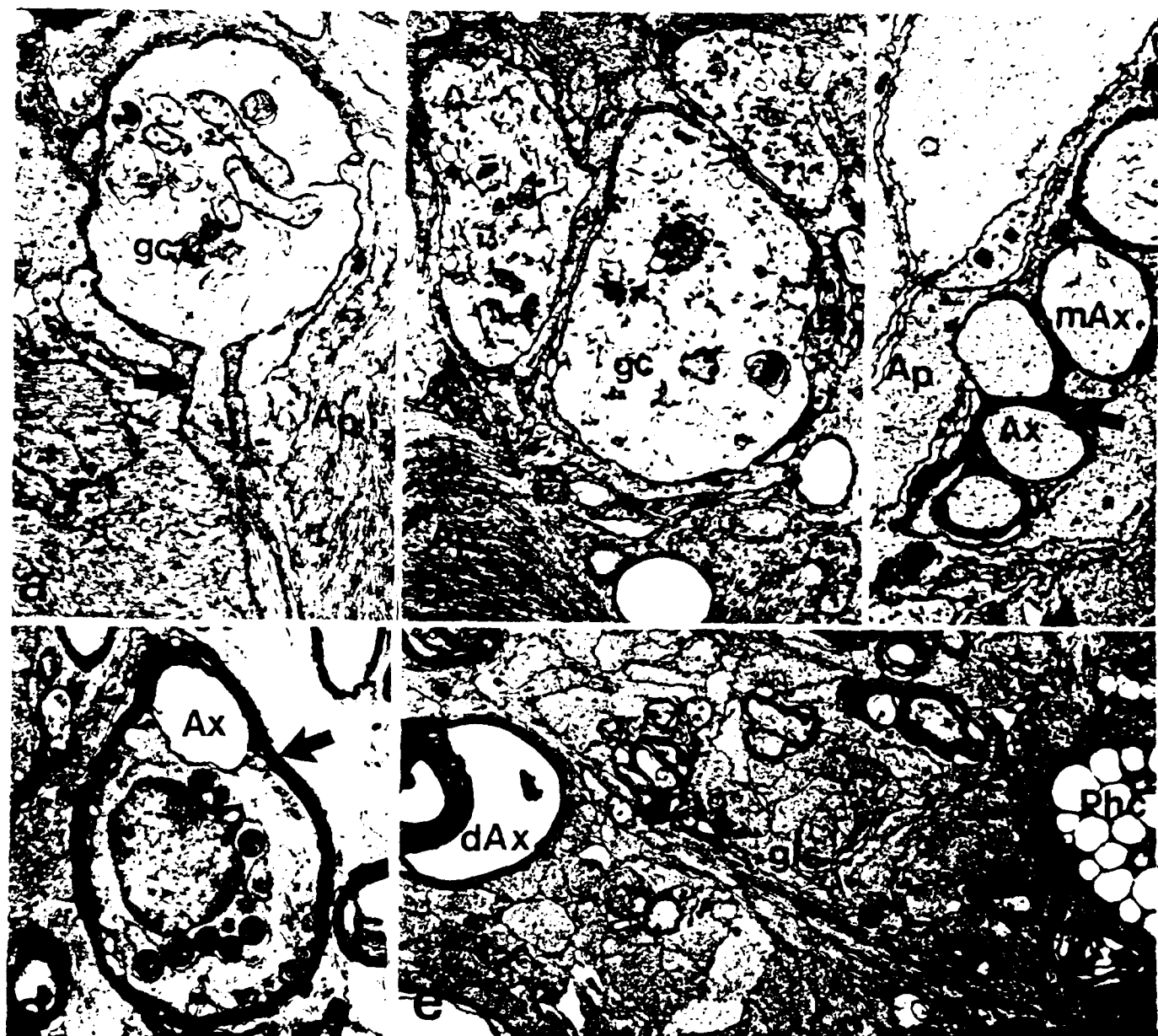


FIGURE 21



FIGURE 22

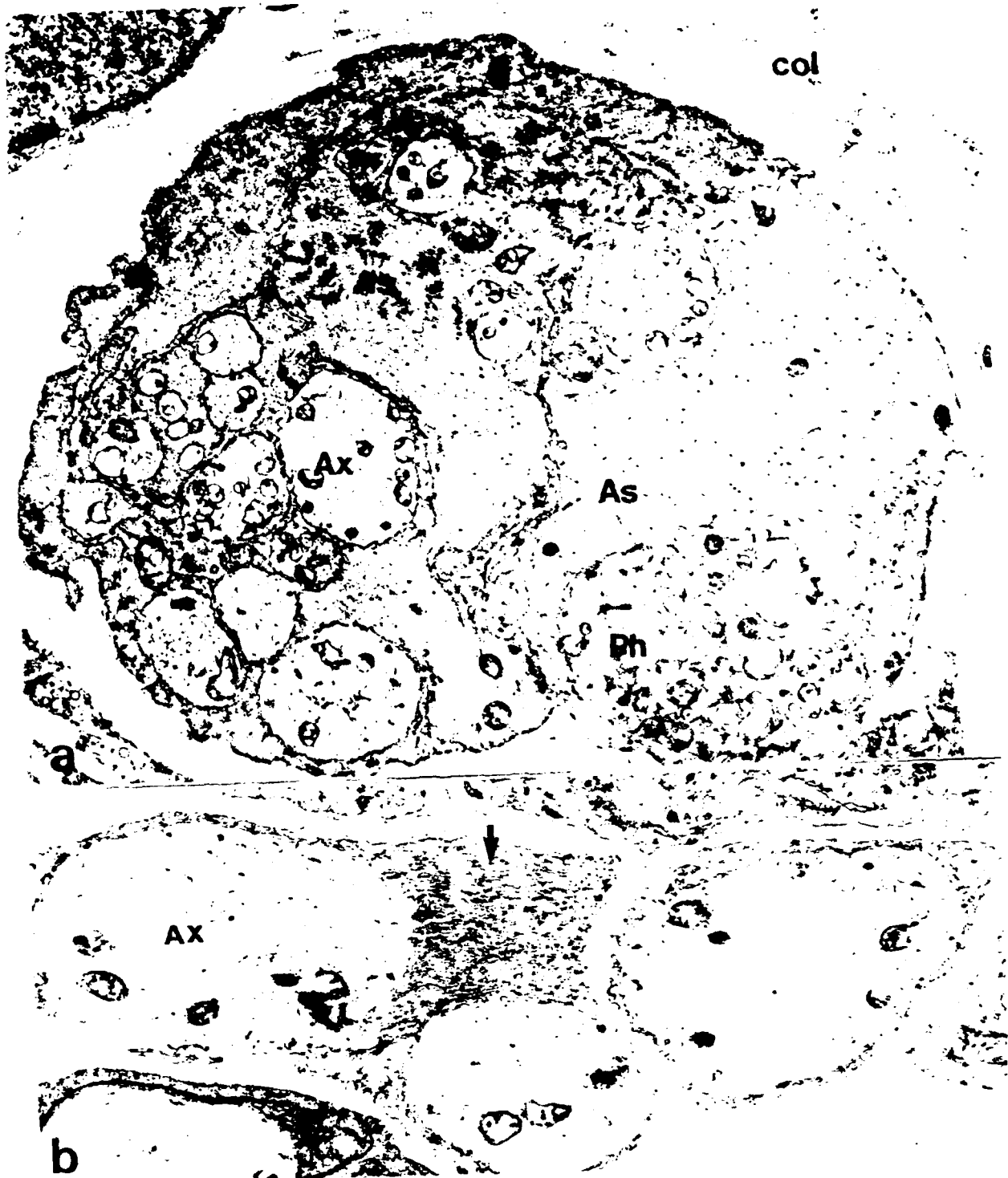


FIGURE 23



FIGURE 24

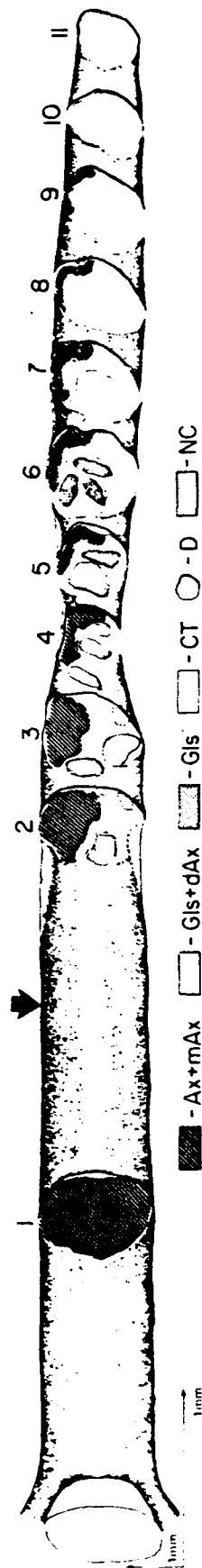
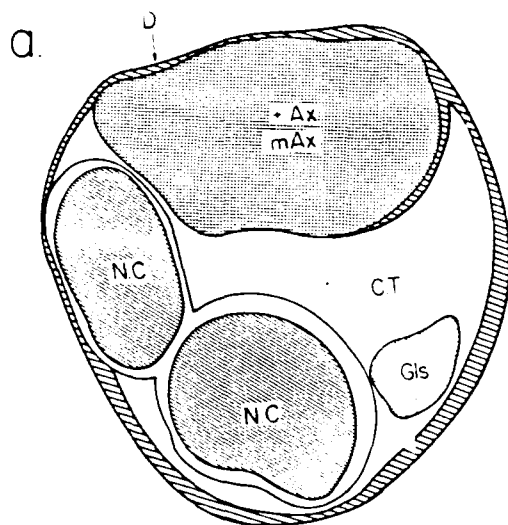
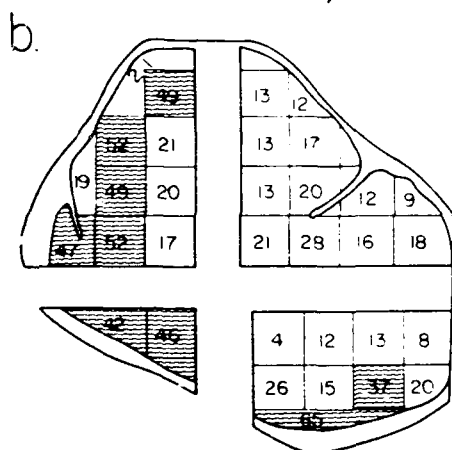


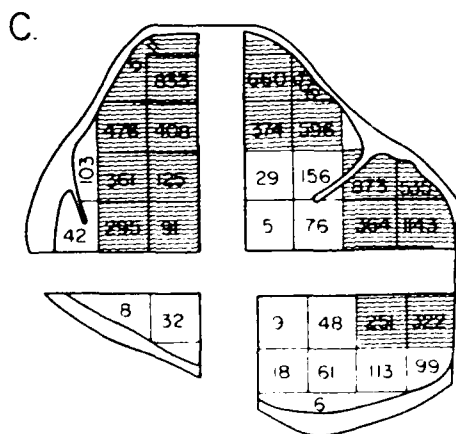
FIGURE 25



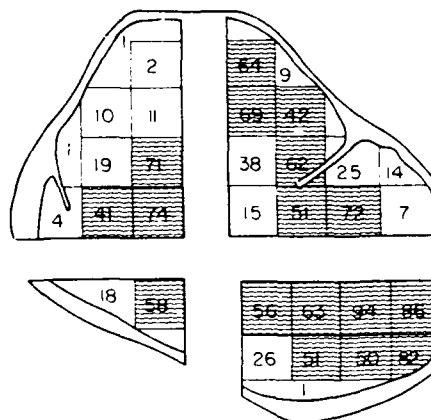
Unmyelinated Axons
mean = $29/100\mu^2$



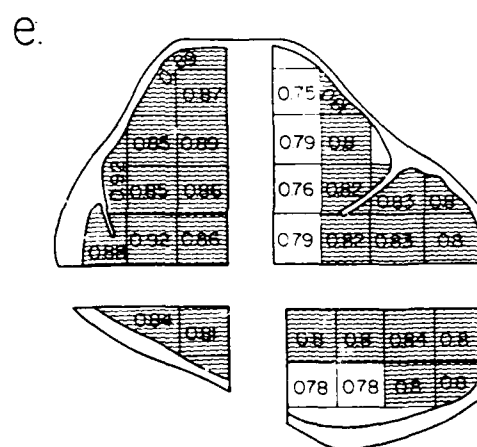
Myelinated Axons
mean = $295/100\mu^2$



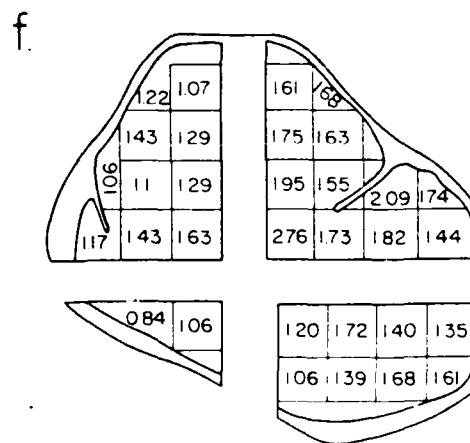
d. mean = $40/100\mu^2$



Myelin index



Myelinated Axons Diameter



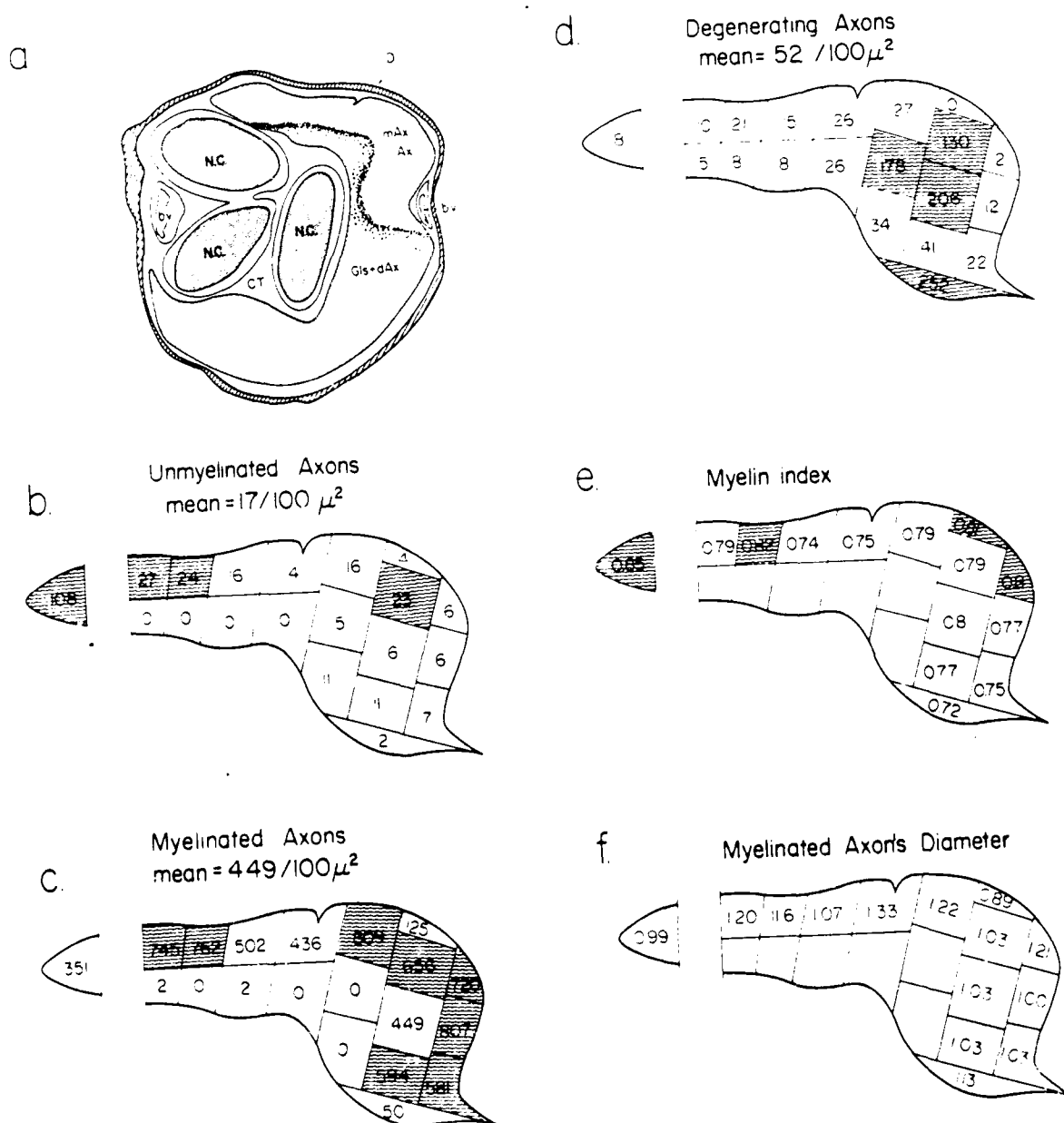


FIGURE 27

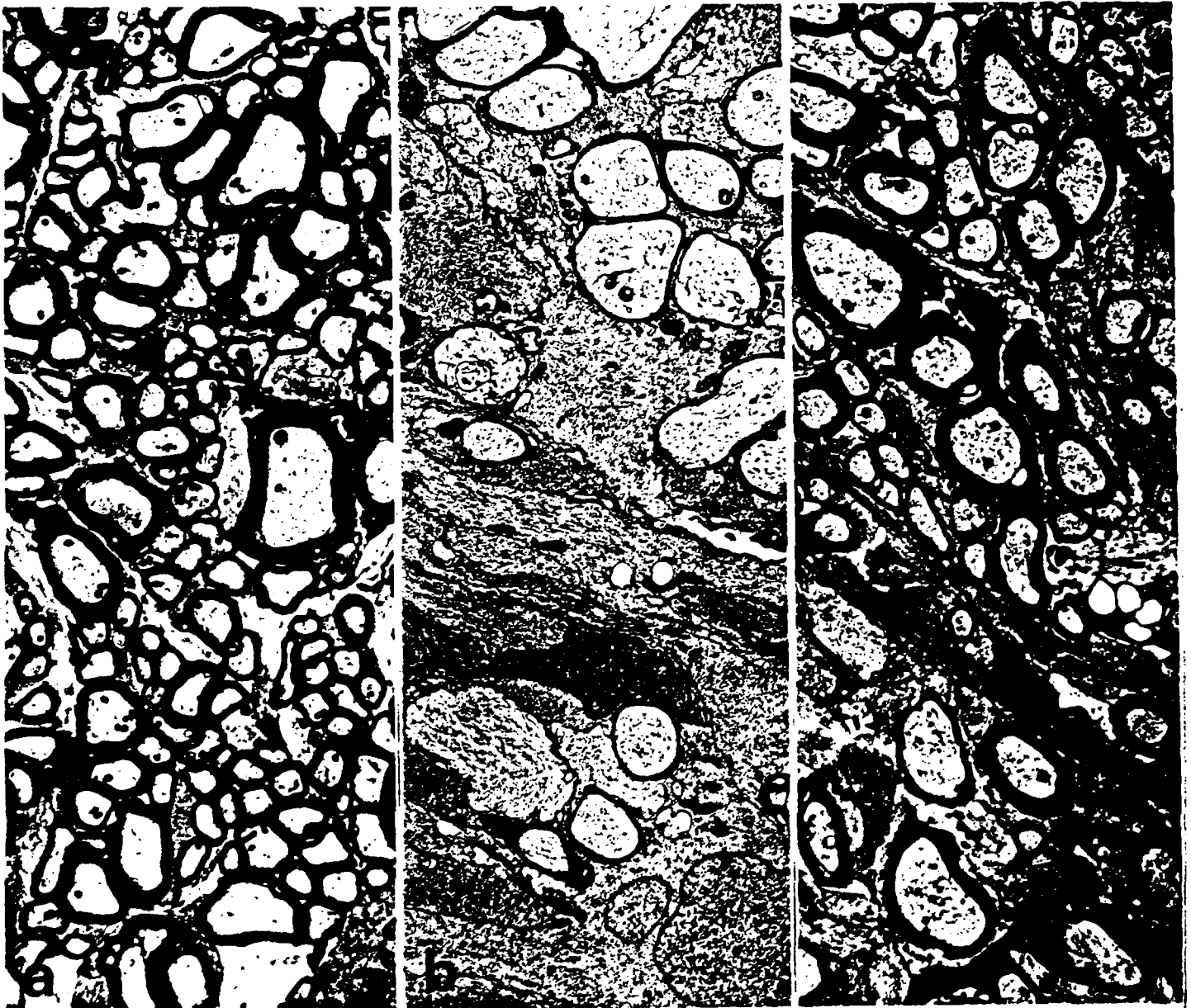


FIGURE 28

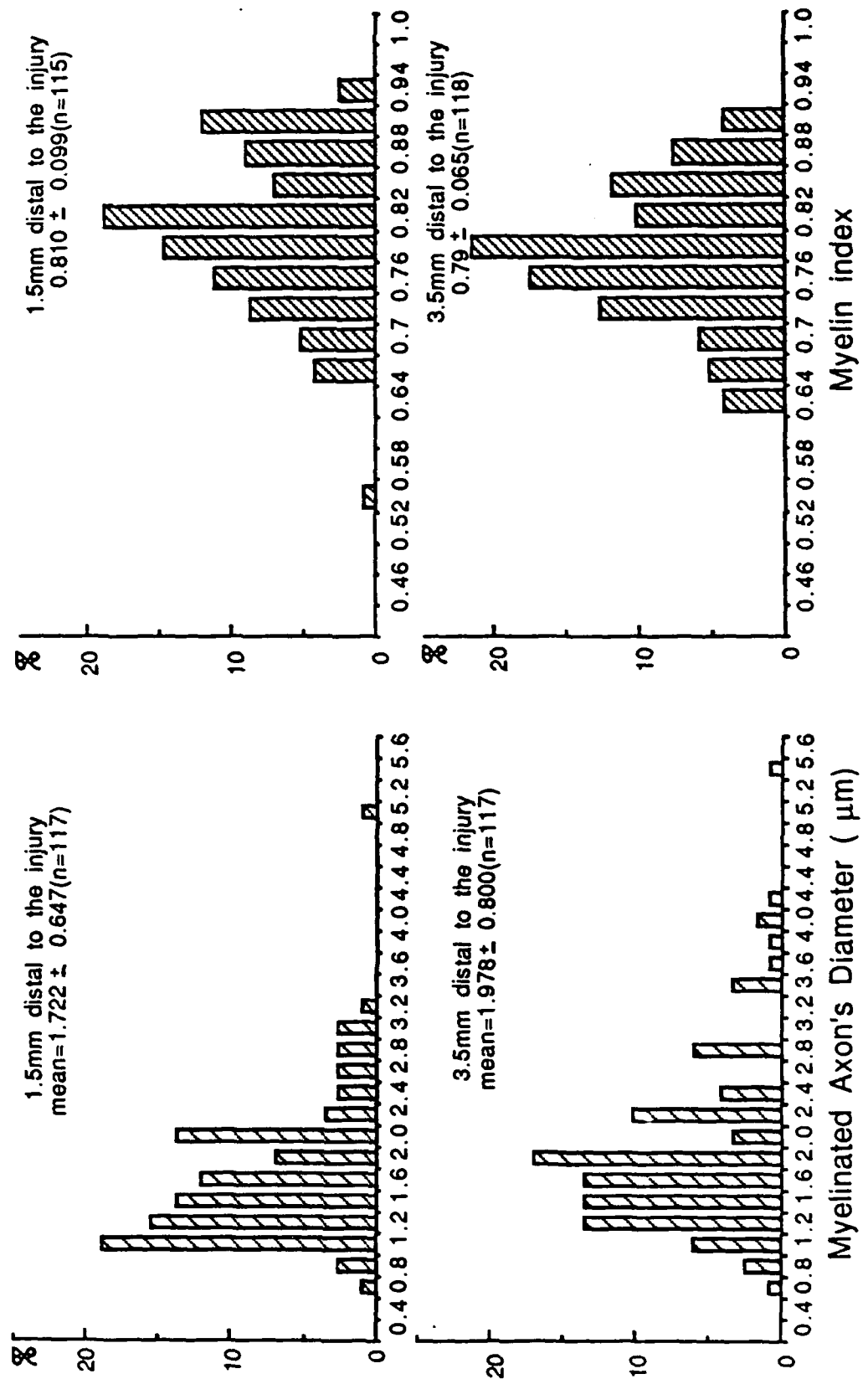


FIGURE 29

